CHARACTERISATION OF A CHITINASE PRODUCED BY AN ENVIRONMENTAL *PAENIBACILLUS CHITINOLYTICUS* STRAIN PBY

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Masters of Science.

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DECLARATION

I declare that the dissertation being submitted is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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Varsha Parshotam Chhiba

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ABSTRACT

An isolated environmental strain PBY when submitted for 16s rDNA analysis was shown to have 99% sequence similarity to *Paenibacillus chitinolyticus* sequence data by BLAST searching the sequence data obtained. The strain when induced in minimal media containing pure chitin was found to produce multiple chitinolytic enzymes by 2D polyacrylamide gel separation with pI's ranging from 4.8 to 5.3 and from 7.0 to 9.2. Further characterisation work done on the dominant extracellular chitinolytic enzyme (chitinase with a pI 7.0) showed that this enzyme is an endochitinase with a preference for longer chain substrates. The K_m values when incubated with 4-MU CHB and 4-MU CHT being were found to be 7.45 µM and 5.72 µM respectively. The same chitinase enzyme when incubated with a trimeric substrate produced a dimer and GlcNAc as final products.

The characterised extracellular enzyme showed optimum chitinase activity between pH 7.0 to 7.5 with 80% activity retained between pH 6.5 and 8.0. The optimum temperature range for the extracellular chitinase was found to be between 30^{o}C and 40^{o}C, while the purified chitinase maintained 80 to 90% of activity at temperatures ranging from 10^{o}C to 50^{o}C after an hour of incubation of the enzyme at the respective temperatures. The characterised chitinase enzyme maintained 50% activity after incubation for an hour at 65^{o}C.

This appears to be the first known *P. chitinolyticus* chitinase enzyme to be purified and characterised to date.

ACKNOWLEDGEMENTS

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This degree is for Niamh and Neharika who remind me daily of all I have to be thankful for. Finally to little Ivan Cameron who I have never met…you have made me see my children in a miraculous light and that will never be forgotten.

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ABBREVIATIONS

(v/v)	Volume to Volume Ratio
(w/v)	Weight to Volume Ratio
2D-PAGE	Two–Dimensional Polyacrylamide Gel Electrophoresis
4–MU	4-Methylumbelliferone
4-MU Glc	4-Methylumbelliferyl Glucosaminide
4-MU CHB	4-Methylumbelliferyl Chitobioside
4-MU CHT	4-Methylumbelliferyl Chitotrioside
Anion EX	Anion Exchange
Bacillus sp.	*Bacillus* species
BSA	Bovine Serum Albumin
Cation EX	Cation Exchange
CFE	Cell Free Extract
CHB	$_{N, N}^{I}$ Diacetylchitobiose
CHP	$_{N, N}^{I}{}_{, N}^{II}{}_{, N}^{III}{}_{, N}^{IV}$ Pentacetylchitopentaose
CHT	$_{N, N}^{I}{}_{, N}^{II}$ Triacetylchitotriose
DMF	Dimethylformamide
DTT	Dithiothreitol
GH	Glycosyl Hydrolase
Glc	Glucosamine
GlcNAc	Beta- N-Acetylglucosamine
hexosaminidase	Beta-N-Hexosaminidase
IPG	Immobilised pH Gradient
kDa	Kilodaltons
K_m	Michaelis-Menten Constant
MES	Morpholino Ethanesulphonic Acid
NaCl	Sodium Chloride
n	Number of data points
^{o}C	Degrees Celsius
P. chitinolyticus	*Paenibacillus chitinolyticus*

rDNA	Ribosomal Deoxyribonucleic Acid
rpm	Revolutions Per Minute
∑	Arithmetic Mean
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
sp.	Singular species within a genus
spp.	Multiple species within a genus
TEMED	$_{N}$, $_{N}^{I}$, $_{N}^{II}$, $_{N}^{III}$-Tetramethylethylenediamine
TLC	Thin Layer Chromatography
VAR	Variance of SD data
V_{max}	Maximal Velocity
Tris	Tris-2-Amino-2-(Hydroxymethyl)-1,3, propandiol
TSB	Tryptone Soy Broth

Standard Chemical Abbreviations were applied in text

IUPAC-IUBMB three and one letter codes for amino acids were used

CHAPTER ONE

LITERATURE REVIEW

1 CHAPTER ONE - LITERATURE REVIEW

1.1 Chitin

Chitin, a high molecular weight linear polymer of β (1, 4) linked *N*-acetylglucosamine residues (GlcNAc), is one of the most abundant renewable resources obtained from marine invertebrates such as crustaceans and protozoa. An estimated 80 000 metric tons of chitin is yearly generated from marine waste alone (Gooday 1990). It is also found in fungi (Adams 2004) and insects, as well as diatoms (Bartnicki-Garcia and Lippman 1982). In the human body GlcNAc is synthesized from glucose and is incorporated into glycoproteins as well as glycoaminoglycans.

The phylogenetic distribution of chitin containing organisms in nature indicates the role that the structural polysaccharide plays in the organism being studied. Prokaryotes show a lack of chitin in their cells despite having peptidoglycan polysaccharide backbones which are similar to chitin. Most fungal cells have some chitin in their cell walls (percentage composition in cell wall ranging from 22 to 44%) (Gohel *et al.* 2006). Chitin, together with other polysaccharides, has been commonly employed in taxonomic studies performed on fungi. In the protists chitin is found present in cysts as well as in the cell wall of some of the ciliates, amoeba, chrysophytes, algae and in the spines of some diatoms (Bartnicki-Garcia and Lippman 1982). Chitin formation in animals is limited to its presence in a few organs such as the integument of arthropods and insects such as *Bombyx mori* (Daimon *et al.* 2005), in the chitinous 'eggshell' of parasitic nematodes such as *Ascaris suum* and *Ascaris lumbricoides* (Geng *et al.* 2002) and in molluscs.

1.1.1 Chitin synthesis in fungal cells

Chitin is crystalline in nature with the tensile strength of the polymer being much stronger than most artificially produced materials. The strength of the polymer is as a result of the extensive hydrogen bonding along the chains during production. The polymer is widely distributed in the fungal kingdom with almost all fungi having significant amounts of chitin in their cell walls.

The synthesis of chitin involves many multifaceted cellular activities which include the biotransformation of simple metabolites and the emergence of the polymer. The catalytic units assemble in the cell membrane therefore allowing the polymerised GlcNAc molecules to be extruded into the extracellular environment (Cohen 1987).

The sequence of chitin formation begins with the hydrolysis of trehalose, followed by the phosphorylation of glucose, transmutation to form phosphorylated fructose, amination, acetylation, and finally conversion of the product to a nucleotide phosphate-acetylated amino sugar. Precursors are also channelled to other alternative biochemical pathways (Cohen 1987). The amination of fructose-6-phosphate to glucosamine-6-phosphate is the critical point for incorporation of metabolites into amino-sugar containing bio-polymers such as glyco-proteins, muco-polysaccharides as well as chitin (Figure 1).

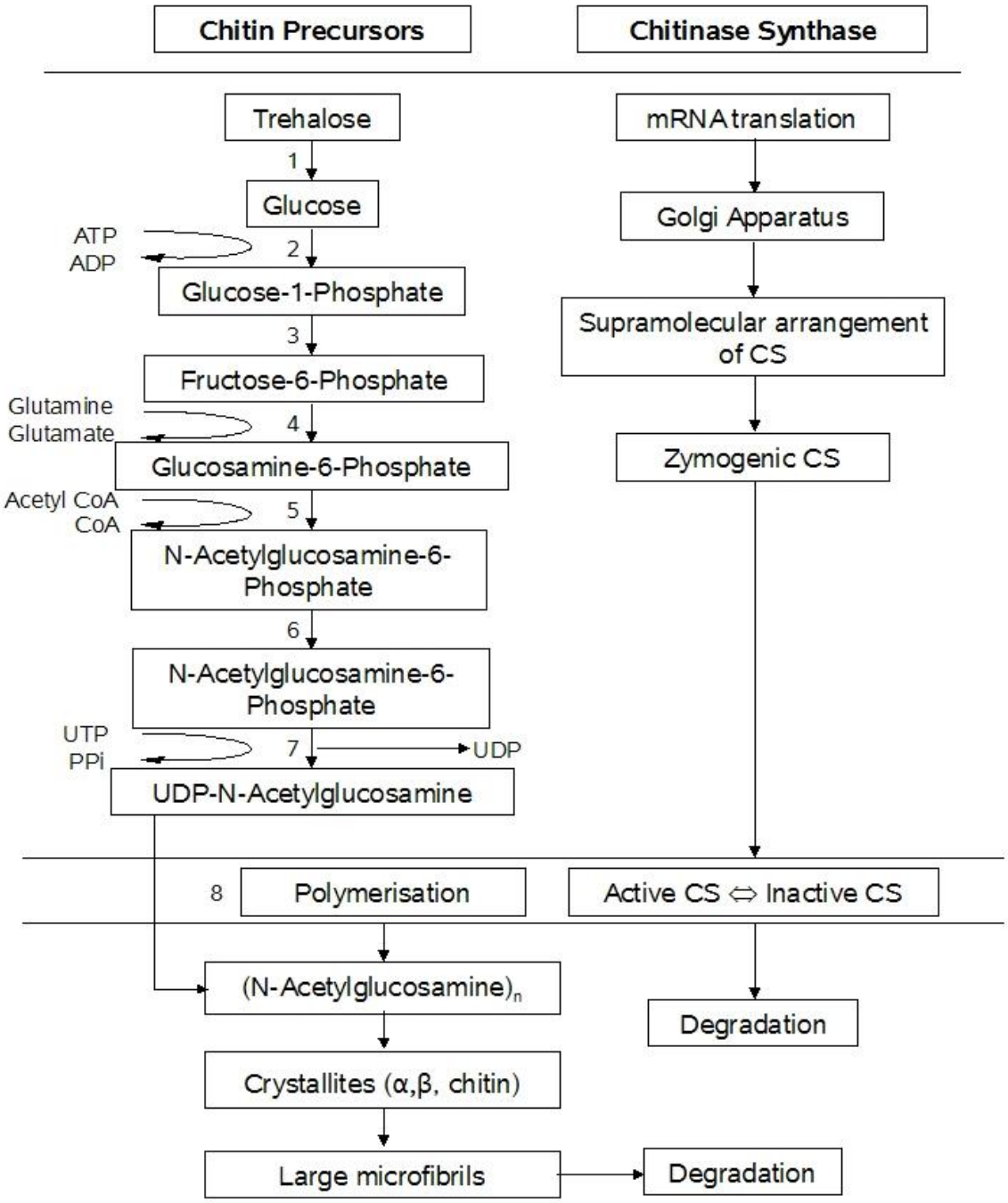


Figure 1. Flow diagram illustrating chitin formation

The following enzyme systems are involved 1: trehalase, 2: hexokinase, 3: glucose-6-phosphate isomerase, 4: glutamine fructose-6-phosphate aminotransferase, 5: glucosamine-6-*N*-acetyltransferase, 6: phosphoacetylglucosamine mutase, 7: UDP-*N*-acetylglucosamine pyrophosphorylase, 8: chitin synthetase. CS–Chitin synthase. Image adapted from (Cohen 1987).

1.2 Chitin degrading enzymes

Chitin degrading enzymes such as chitinases, chitosanases, β–*N*–acetylhexosaminidase (hexosaminidase) and chitin deacetylase enzymes are gaining importance for their biotechnological applications. These include the use of chitinases in the agricultural sector for control of plant pathogens. The estimated loss of crops due to plant diseases caused by these pathogens is said to be in the region of 25% of total yield in Western countries and as much as 50% loss in most developing countries (Gohel *et al.* 2006). Approximately a third of these are as a result of fungal infections alone. Commercial fungicides, which have previously been employed in plant pest control strategies, have lost favour in the agricultural sectors due to their adverse effect on the environment, as well as their nonspecific toxicity. A large number of chemical pesticides have also been shown to be persistent in nature with elevated concentrations of these toxic compounds being found in groundwater and runoff water samples. Current practices in molecular biology such as generating transgenic plant cell lines with increased resistance to pathogens have been developed, however even these have been shown to be susceptible to certain pathogens with the lack of genetic diversity in these plant lines being a major drawback.

Chitinolytic enzymes are synthesized by microorganisms as a means to hydrolyse chitinolytic material. This trait could be manipulated to offer a "greener" approach to the control of plant associated fungal as well as nematode pathogens. Chitinases are known to release oligo *N*-acetyl glucosamine's which function in plants to elicit the activation of a defence related response at the cellular level in the plant cells (Kasprzewska 2003). The form of expression, which can be systematic or local, is dependant on the infecting pathogen, its virulence, and the particular chitinase class (Meier *et al.* 1993). This two-fold strategy of pathogen elimination and plant protective stimulation could be the environmental strategy best able to satisfy both environmental and agricultural needs.

Chitinolytic enzymes have also been applied in human therapeutic studies with chitin residues such as pentacetylchitopentaose (CHP) as well as hexaacetylchitohexaose showing antitumor activity (Patil *et al.* 2000). The dimeric substrate, diacetylchitobioside (CHB) has been widely used as a starting material for the synthesis of biologically active compounds (Terayama *et al.* 1993). Kobayashi *et al*. (1996) employed a *Bacillus* chitinase in the generation of both regio and stereoselective glycosidic bonds between chitobioside units. The artificial chitin produced in this manner has found application in numerous scientific studies and in the production of multifunctional substances (Kobayashi *et al.* 1996).

1.2.1 The group of glycosyl hydrolase enzymes

Chitinases (EC 3.2.1.14), chitosanases (EC 3.2.1.132), and β-*N*-hexosaminidase (hexosaminidase) (EC 3.2.1.52) belong to the group of Glycosyl Hydrolase (GH) enzymes (EC 3.2.1.x) which are primarily responsible for carbohydrate metabolism (Henrissat 1991). Following the International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature the first three digits indicate that the enzymes hydrolyse *O*-glycosyl linkages with the last digit providing information pertaining to the nature of the substrate being hydrolysed or the mechanism of activity (Henrissat 1991). All GH enzymes are documented to act by a general acid catalysis mechanism, in which two amino acid residues participate in a single or double displacement reaction resulting in either inversion (equatorial to axial configuration change and vice-versa) or a retention of configuration (equatorial substrate to equatorial product, or axial substrate to axial product) at the anomeric carbon of the substrate as shown in Figure 2 (Sinnott 1990).

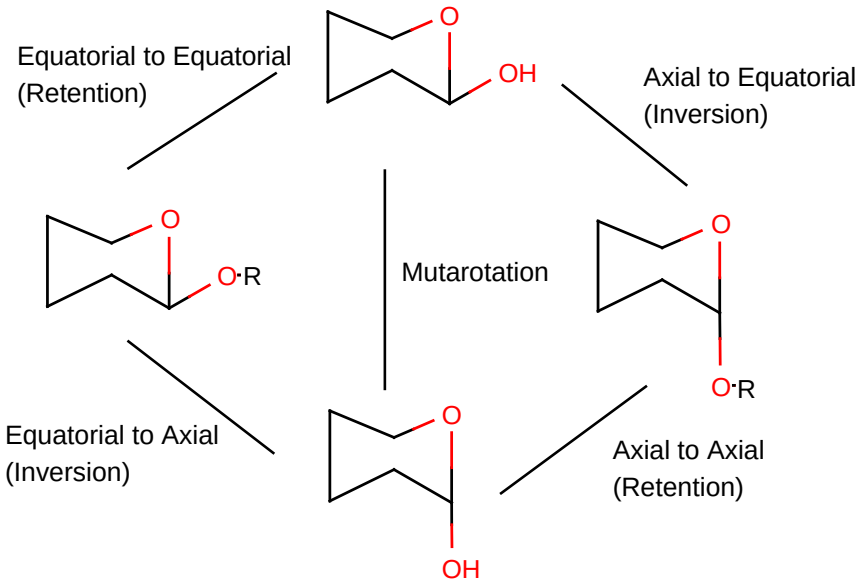


Figure 2. Pyranoside hydrolysing enzyme

Axial (a) or equatorial (e) glycosidic bonds and the configuration of the generated products. Image adapted from (Henrissat and Davies 1997).

GH enzymes have developed a number of ways to lower the energy barrier needed for hydrolytic action. A proposed strategy is the distortion of the substrate to a half chair conformation. Protonation of the glycosidic bond is accompanied by the lengthening of the bond therefore favouring binding of the elongated transition state compound over that of the ground state substrate (Strynadka and James 1991).

Many GH enzymes have a modular structure consisting of a catalytic domain and one or more non-catalytic domains which may or may not be involved in substrate binding. Among all bacterial chitinases sequenced to date, no relationship has been found with regards to percentage similarity between the catalytic and chitin-binding domains from pairwise comparisons work done. The chitin binding domain seems to be evolutionarily conserved among bacterial chitinases (Svitil and Kirchman 1998).

GH enzymes exist as mono-specific or poly-specific enzymes. This by definition implies that the enzyme has the ability to hydrolyse a single (mono-specific) or multiple related substrates (poly-specific). The presence of poly-specific enzyme systems is largely a result of evolutionary divergence with gene duplication commonly observed amongst GH enzyme producing strains. In strains demonstrating gene duplication, the original gene would continue to hydrolyse the original substrate whereas the duplicated gene would constitute the formation of enzymes with specificity directed towards varied but structurally similar substrates (Henrissat 1991).

Detailed primary structure analysis of the GH enzyme families have resulted in the identification of potential glutamate and aspartate active site residues (Henrissat and Bairoch 1993), with site directed mutagenesis data supporting the predictive work. Site directed mutagenesis and crystallographic data from family 18 chitinases have shown that a conserved glutamate is involved in the catalytic mechanism and probably acts as a proton donor (Watanabe *et al.* 1993; (Perrakis *et al.* 1994). Van Aalten *et al*. (2007) proposed a catalytic mechanism involving three critical catalytic residues, namely Asp^{140}, Asp^{142} and Glu^{144}, in an exochitinase produced by *S. griseus* (Van Aalten *et al.* 2007). The resting enzyme showed the Asp^{142} residue was spatially separated from the Glu^{144} whereas binding of the substrate causes distortion of the pyranose ring structure of the substrate to a skewed boat conformation and rotation of the Asp^{142} towards Glu^{144}, therefore enabling hydrogen bonding between the hydrogen of the acetamido group (Figure 3). Resolved enzyme-substrate crystal structures reported by the same authors showed the chitin oligomer spanning the catalytic site of a multidomain chitinase (CHP) in the presence of *Serratia marcescens* ChiB chitinase.

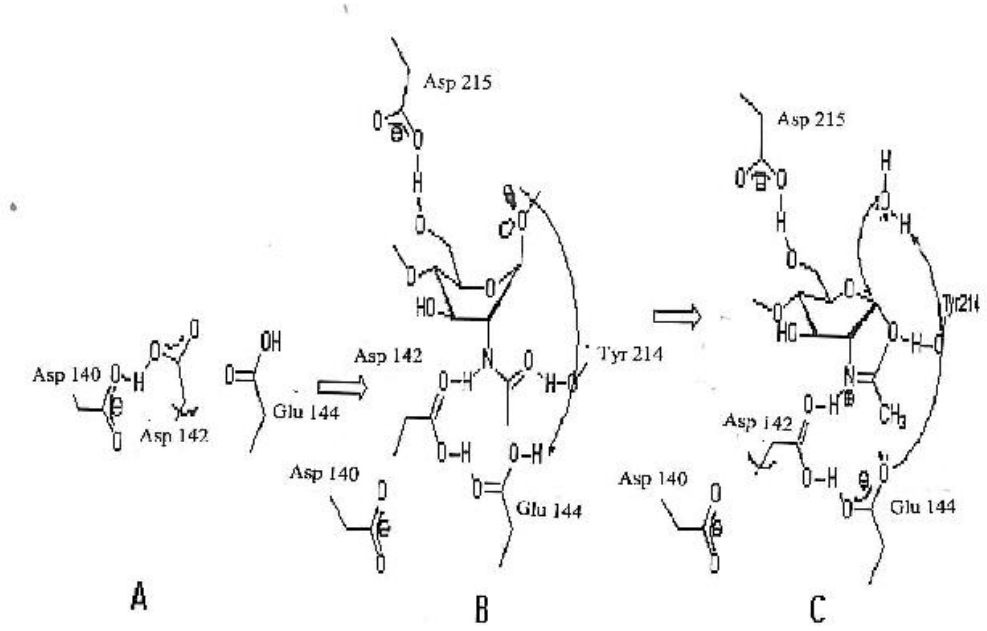


Figure 3. Role of conserved Asp and Glu catalytic residues in most family 18 chitinases

The role of Asp^{140}, Asp^{142} and Glu^{144} during separate stages of chitin catalysis in *S. griseus* is shown. (*A*) In the resting enzyme state Asp^{142} is too far away to interact with Glu^{144}, however binding of the substrate causes a conformational change of the pyranose ring to a boat or skewed boat conformation. (*B*) The rotation of Asp^{142} toward Glu^{144} enables hydrogen bond interaction between the hydrogen of the acetamido group, Asp^{142} and Glu^{144}. (C) Hydrolysis of the oxazolinium ion intermediate leads to protonation of Glu^{144} and rotation of Asp^{142} to its original position. Image adapted from (Van Aalten *et al.* 2007).

1.2.2 Chitinase enzymes (3.2.1.14)-Comparison of family 18 and family 19 GH enzymes

Chitinase enzymes which are classified as either belonging to family 18 or family 19 of the GH group of enzymes are an important subsection of an array of chitinolytic enzymes produced in nature. They allow for the biological hydrolysis of chitin without the necessity for use of harsh chemicals and conditions and allow for the generation of chito-oligosaccharides which have potential for use in the production of therapeutic compounds.

Chitinase family classification is based on the individual mode of hydrolysis of the enzyme. Chitinases in general are random splitting hydrolases that recognise the *O*-glycosyl linkage between chito-saccharide residues for catalysis. Endochitinases split chitin polymers into oligosaccharides by hydrolysing internal *O*-glycosyl linkages as show in Figure 4, while exochitinases function by splitting only terminal GlcNAc residues or dimers from the reducing end of the chitin polymer.

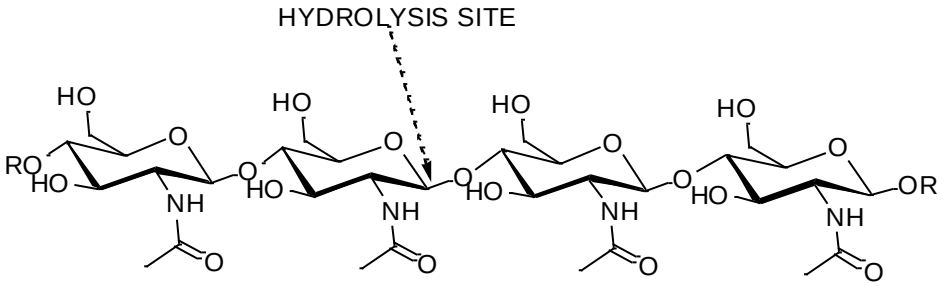


Figure 4. Mechanism of hydrolysis by endochitinase enzymes

Endochitinase enzymes release dimers or multimers from the non-reducing end of the large multimeric substrate. The arrow depicted in the illustration indicates the hydrolysis site typified by the presence of an *O*-glycosyl linkage. The R indicates polymer continuity. Image adapted from (Brameld *et al.* 1998).

Substrate hydrolysis by chitinase enzymes can either result in an inversion or retention of configuration of the anomeric carbon. The anomeric configuration of the product (α or β) depends on whether the chitinolytic enzyme involved functions via a substrate assisted (family 18) or acidic (family 19) catalytic mechanism. Both GH family enzymes also differ in their individual chitinase activity inhibitors (Table 1). In both substrate inversion as well as retention cases the position of the proton donor is the same, i.e. the donor is within hydrogen bonding distance of the glycosidic oxygen. The difference in mechanism is attributable to the distance between the nucleophilic catalytic base and the anomeric carbon of the substrate. The average distance in retaining enzymes is ~5.5 Å versus ~10 Å in inverting enzymes (McCarter and Withers 1994).

Table 1. Comparison between family 18 and family 19 GH enzymes

GH Family	Catalytic mechanism	Intermediate	Anomeric configuration of product	Chitinase inhibitors	Reference
18	substrate assisted	oxazolinium ion	β	allosamidin argadin	(Fukamizo 2000)
19	acidic	oxocarbenium ion	α	amidines amidrazones	(Fukamizo 2000)

Family 18 chitinases are suggested to act in a retaining mechanism, where the water ingress to perform hydrolysis results in the formation of an oxazoline ring on the substrate (between the carbonyl oxygen of the *N*-acetyl group and C-1 of the GlcNAc). With the formation of the oxazoline ring and the absence of steric hinderance, the water is thought to enter from the other side to form the β-anomer and thus retain the configuration at C-1 (Koga *et al.* 1999) (Figure 5).

In family 18 chitinases, only one carboxylate functions as a catalytic carboxylate. The binding cleft of family 18 chitinases takes place through a substrate assisted mechanism. Hen egg white lysozyme (HEWL) and barley-chitinases are capable of hydrolysing related polysaccharides and have a binding cleft represented by (-4)(-3)(-2)(-1)(+1)(+2) whereas the binding cleft of family 19 chitinases is represented by (-3)(-2)(-1)(+1)(+2) (+3) (Fukamizo 2000). Note that substrate binding subsites in GH enzymes use the *–n* to *+n* nomenclature where *n* is an integer representing the monomeric sugar resides in the substrate polymer. Subsites labelled as *–n* represents the non-reducing end of the substrate and *+n* the reducing end. Cleavage takes place between the -1 and +1 subsites (Davies *et al.* 1997).

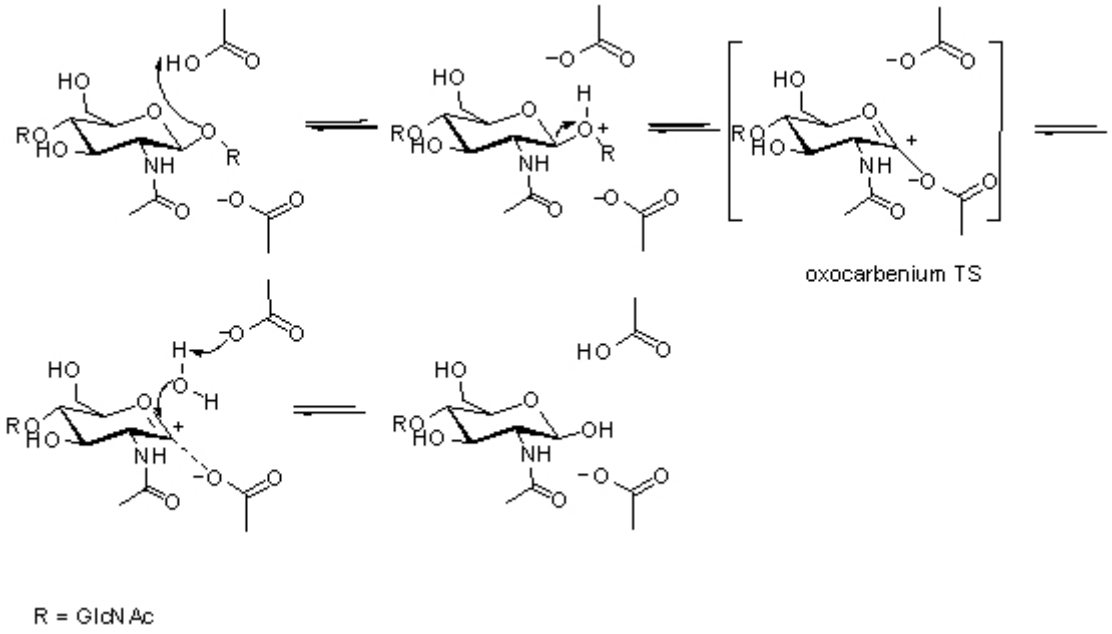


Figure 5. Double displacement mechanism of chitinases showing retention of product configuration as demonstrated in family 18 GH enzymes

A double displacement mechanism leads to the retention of configuration of the anomeric carbon on the sugar residue undergoing biocatalysis. The two catalytic carboxylates involved in the reaction are located on opposite sides of the sugar plane. The protonation of the GlcNAc residue in a boat conformation leads to an oxazoline intermediate which is hydrolysed to yield a product with an α-anomeric configuration. Image adapted from (Dahiya *et al.* 2006).

Family 19 chitinases (also known as class ^{I}, class ^{II}, class ^{III}, class ^{IV}, class ^{V}, class ^{VI}) are enzymes largely produced by plants that function in plant defence against fungal and insect pathogens by destroying their chitin containing cell wall. Family 19 chitinases function through an inverting mechanism. Within this class of chitinases, steric hinderance prevents the formation of the oxazoline ring structure and in such a case the configuration is inverted to an α-anomer thus accommodating the formation of the oxazoline ring (Koga *et al.* 1999) (Figure 6).

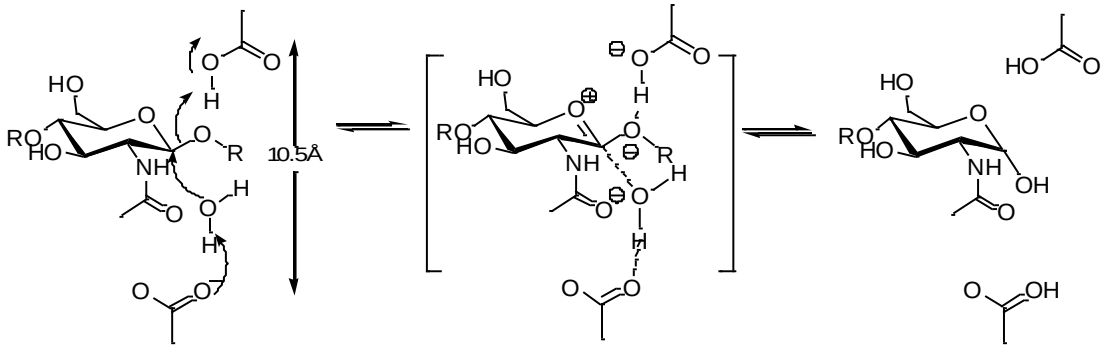


Figure 6. Inversion of configuration of family 19 GH enzymes

Illustration of an inverting GH enzyme (family 19 GH enzyme) with a β-substrate being converted to a product with an α-anomeric carbon configuration. The catalytic machinery involves two catalytic carboxylates that enable a general acid-catalysed leaving group to depart 2) followed by a general base assistance to nucleophilic attack by a single water molecule. Image adapted from (Dahiya *et al.* 2006).

Chitinase catalytic mechanism and molecular structure data

Bacterial chitinases are separated into three major subfamilies (A, B, C) based on the respective amino acid sequences of the individual catalytic domains (Watanabe *et al.* 1993). Subfamilies A chitinases have a third domain corresponding to the insertion of an α + β fold region between the seventh and eighth $(\alpha/\beta)_8$ barrel whereas chitinases belonging to Subfamily B and C do not have this insertion.

The crystal structure of chitinase from *Serratia marcescens* (a family 18 chitinase) shows the presence of $(\alpha/\beta)_8$ barrel structure (Perrakis *et al.* 1994) with similar structures observed in *Hevea brasiliensis*, endo-β-*N* acetylglucosaminidases F1 and H from *Flavobacterium meningosepticum* (Terwisscha van Scheltinga *et al.* 1994) and in *Bacillus circulans* WL-12 (Watanabe *et al.* 1993). On the other hand, family 19 chitinases from barley seeds have a different fold from that of family 18 enzymes; the fold is similar to that of goose egg white lysozyme (GEWL) and comprise of two helices and three stranded β-sheets (Monzingo *et al.* 1996); (Honda and Fukamizo 1998). The substrate binding and catalytic core of family 18 chitinases consists of three stranded β-sheets and two alpha-helices with family 18 chitinases consisting of several functional domains, namely the catalytic domain, a fibronectin type III-like domain as well as a chitin binding domain (Figure 7). The fibronectin domain was found to play a

significant role in the hydrolysis of insoluble chitin containing substrates such as colloidal chitin, but did not affect chitin binding by the enzyme (Watanabe *et al.* 1993).

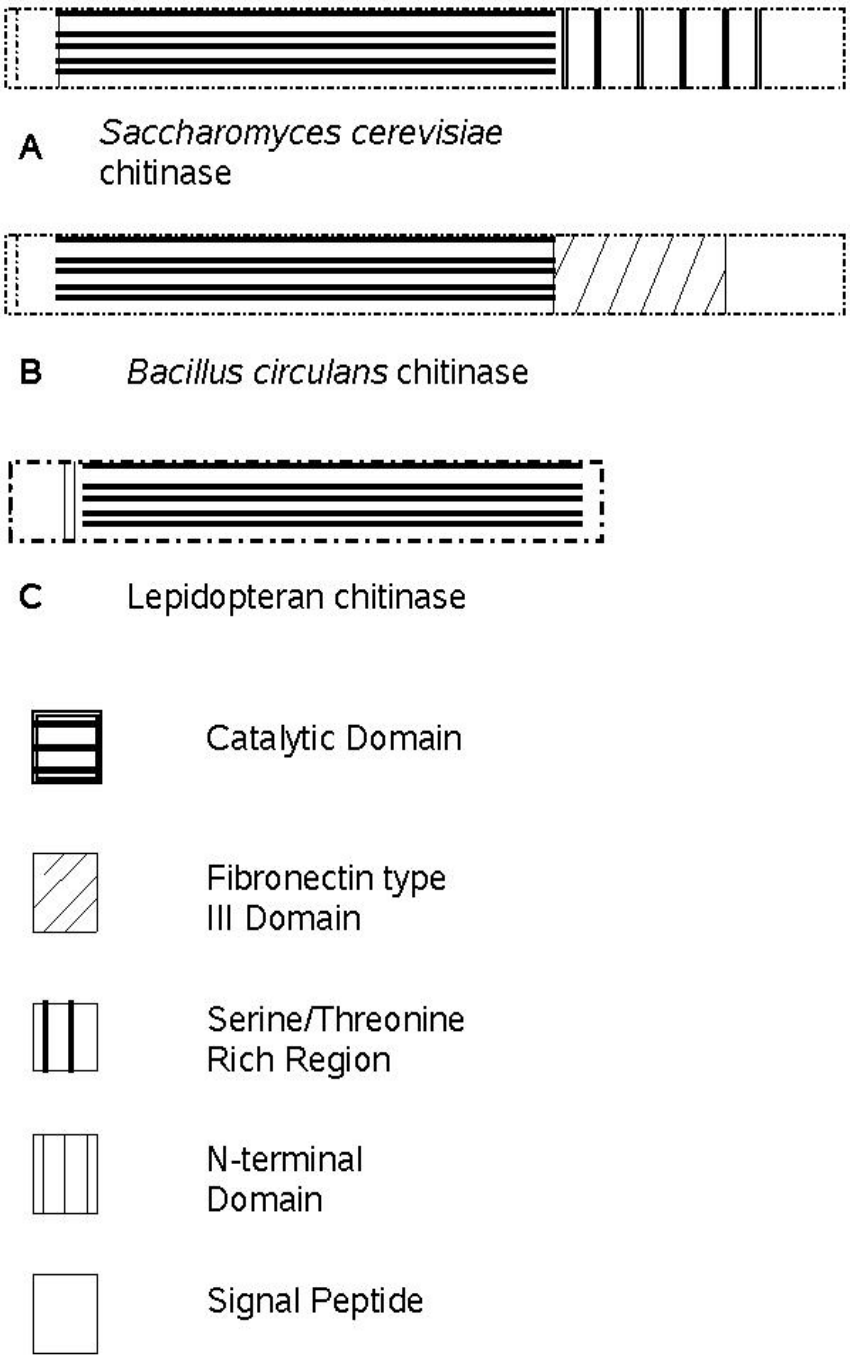


Figure 7. Schematic illustration of varied chitinase domain structures

Image adapted from (Patil *et al.* 2000; Daimon *et al.* 2005)

Family 19 chitinases (also known as class ^{I}, class ^{II}, class ^{III}, class ^{IV}, class ^{V}, class ^{VI}) are enzymes largely produced by plants that function in plant defence against fungal and insect pathogens by destroying their chitin containing cell wall. Class ^{I} and class ^{II} chitinase enzymes both have amino acid sequence data that is >50% identical to tobacco class ^{I} and class ^{II} chitinase sequence data with a conserved N-terminal domain. They do however differ in their presence or absence of a cysteine-threonine rich domain which functions as an acceptor site allowing *O*-glycosylation to take place (Raikhel and Lee 1993). Chitinase class ^{III} enzymes have amino acid sequence data that has >30% sequence homology to class ^{III} tobacco chitinase/lysozyme enzymes with no sequence similarity to class ^{I} and class ^{II} tobacco enzymes (Lawton *et al.* 1992). Plant chitinase enzymes belonging to class ^{IV} show 50% amino acid sequence homology when compared to the *Phaseolus vulgaris* PR4 chitinase. Class ^{IV} chitinase enzymes also have no lectin domain with several deletions of ~22 amino acids in the catalytic domain as well as a truncated C-terminal end (Margis-Pinheiro *et al.* 1991). Chitinase enzymes belonging to class ^{V} have >50% amino-acid sequence homology when compared to sequence data from *Urtica dioica* (stinging nettle) lectin precursor and have a duplicated N-terminal domain (Lerner and Raikhel 1992), and finally chitinase enzymes belonging to class ^{VI} have >50% amino acid sequence homology when compared to a tobacco endochitinase, and shows sequence similarity to many bacterial exochitinases (Melchers *et al.* 1994).

Chitinase enzyme active site structure topologies include pocket or crater structures, the presence of a cleft or groove, or the tunnel active site conformation. The pocket or crater topology is optimal for the recognition of a non-reducing saccharide at the extremity of the substrate. The open structure of the cleft or groove active site conformation allows for the random binding of several sugar units in polymeric substrates and is found in endo-acting polysaccharides such as lysozymes, endocellulases, chitinases, α-amylases and xylanases. Finally the tunnel topology arises when the cleft groove is modified to generate long loops that cover part of the cleft. The resultant tunnel active site topology can therefore accommodate large polymeric substrates which are threaded through the active site and progressively catalyzed (Davies and Henrissat 1995).

Chitinase producing organisms and their functional properties

Chitinase activity has been discovered in a number of varied organisms. In bacteria, chitinases play a role in nutrition and parasitism, whereas in fungi, protozoa and invertebrates, chitinase enzymes are involved in morphogenesis (Merzendorfer and Zimoch 2006). Chitinases are also involved in the defence mechanism of plants (Kasprzewska 2003), and in vertebrates such as humans where chitotriosidase activity helps in defence against nematodal infection. The *Chlorella* virus CVK2 (whose host is unicellular green algal *Chlorella* cells) is an example of a virus capable of producing both chitosanase and chitinase activity that enables viral host cell wall penetration during the lytic cycle as well as viral load release during cell lysis (Yamada *et al.* 1997).

Most family 18 chitinase enzymes are produced by prokaryotes such as *Bacillus* spp., *Serratia* spp., and *Streptomyces* spp. (Tsujibo *et al.* 2000); while chitinase producing plants include some such as *Cucumis melo* sp. (Balde *et al.* 2006), *Pisum sativum* sp. (Mauch *et al.* 1988), *Phaeolus vulgaris* sp. (Boller and Vogeli 1984), and *Nicotiana tabacum* sp. (Suarez *et al.* 2001); fungi such as *Aphanocladium album* sp. (Srivastava *et al.* 1985), *Rhizopus oligosporus* sp. (Yanai *et al.* 1992), and *Saccharomyces s*p. (Cabib *et al.* 1992). Chitinases from nematodes and insects such as *Bombyx mori* also fall within the same family of enzymes (Daimon *et al.* 2005).

A review on microbial chitinase enzymes provide a range of functional parameters that these enzymes can function at or characteristics that they display. In relation to enzyme size, chitinases found in bacteria and fungi are documented to be in the range of 30 to 120 kilodaltons (kDa) with their pI values ranging from 3.5 to 8.8 (Koga *et al.* 1999). The optimum pH of microbial chitinases is said to range between pH 3.5 to 8.0, (however chitinase enzymes have shown altered pH profiles when tested against varied substrates) (Koga *et al.* 1999). Chitinase enzyme stability appears to be directly related to the size of the enzyme, hence smaller compact chitinase enzymes appear to be thermally more stable than their larger counterparts. Finally the production of chitinolytic enzymes in bacteria is generally induced by the presence of chitin in the media (Monreal and Reese 1969); (Svitil *et al.* 1997), with diffusion of chitinases in an

organic matrix said to be possibly affected by the chitin binding domain (Svitil and Kirchman 1998).

Chitinase activators and inhibitors

Chitinases from different GH families are inhibited or activated by varied biological agents as well as metals. In general chitinases are induced by the presence of soluble chitodextrins (Monreal and Reese 1969).

The use of general biochemical methods as well as molecular modelling on hevamine (family 18 chitinase produced by *H. brasiliensis*) found that the oxazoline ion intermediate was stabilised successfully by a neighbouring C-2 acetamido group. The acetamido carbonyl oxygen atom is said to form a covalent bond with the C-1 of GlcNAc and has a transferred positive charge from the pyranose ring onto the acetamido nitrogen atom (Terwisscha van Scheltinga *et al.* 1994).

Allosamidin which is produced by a *Streptomyce*s sp. (Figure 8) was reported to be a specific inhibitor of family 18 chitinases and was said to resemble the oxazoline ring formed between the carbonyl oxygen of the *N*-acetyl group and the C-1 of GlcNAc during hydrolysis (Koga *et al.* 1987). From a structural viewpoint allosamidin looks similar to GlcNAc but lacks a pyranose ring oxygen. Allosamidin contains an oxazoline ring structure in which the methyl group is replaced by a dimethylamine group.

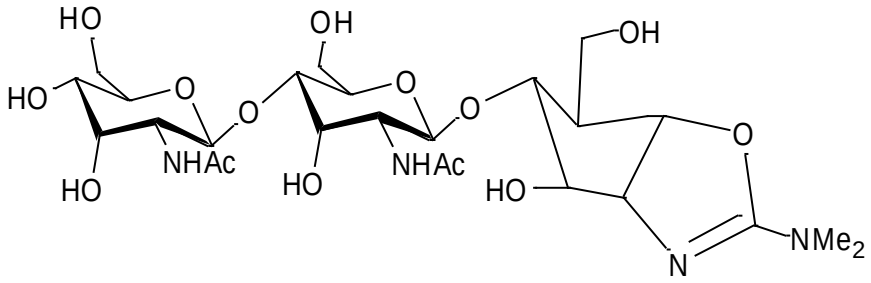


Figure 8. Structure of family 18 chitinase inhibitor namely allosamidin

Image adapted from (Koga *et al.* 1987).

A compound, argadin, (cyclo-*N*-omega-acetyl-L-arginyl-D-prolyl-homoseryl-histidyl-L-2-aminoadipyl) which was isolated from a fungal strain of *Clonostachys* sp. strain FO-7314 was found to inhibit a *Lucina cuprina* chitinase and resulted in cockroach larvae moulting being arrested after injection into the ventral abdominal cavity of the insect (Arai *et al.* 2000).

The presence of certain metal ions serve to inhibit and enhance chitinase activity in certain strains. Divalent metals such as Hg^{2+} and Ag^{2+} commonly inhibit chitinase activity. In the case of Cu^{2+}, there are two chitinases produced by *Alcaligenes xylosoxydans* and *Serratia plymuthica* that are inhibited by its presence (Frankowski *et al.* 2001); (Vaidya *et al.* 2003). Chitinases that are enhanced by the presence of copper include those produced in fish as well as micro-organisms such as *Pseudomonas aeruginosa*.

Finally chitinase repression at a transcriptional level was found to occur when cells of *Trichoderma harzianum* as well as *Streptomyces lividans* were cultured in media containing glucose (Saito *et al.* 1998); de la Cruz *et al*. 1993; Saito *et al*. 1998)

Chitinase transglycosylation activity

Some GH enzymes can catalyse glycosidic bond formation under specific reaction conditions by transglycosylation. This occurs when the positively charged glycosyl intermediate that reacts with water during a normal hydrolysis reaction reacts instead with an incoming alcoholic oxygen from a second sugar molecule during a synthesis type reaction. The product of the synthesis or transglycosylation reaction is the generation of larger molecular weight products then were used in the reaction.

Molecular modification of critical residues within the chitin binding domain of chitinase A from *Serratia marcescens* showed an improvement in transglycosylation activity resulting from an alanine substitution at the Trp^{167} (Aronson *et al*. 2006). A six-fold increase in activity was observed by an aromatic amino-acid being replaced by a non-aromatic small amino acid substitution. This is most likely due to enhanced retention of the glycosyl fragment resulting from an increased stabilization of a Glu^{313} residue which

under normal substrate hydrolysis conditions moves towards the Asp^{315} residue to allow for glycosyl fragment release.

Other chitinolytic enzymes demonstrating transglycosylation activity include a chitinase from the culture filtrate of *Nocardia orientalis* IFO 12806, which accumulated $GlcNAc_7$ as well as $GlcNAc_3$ when incubated with $GlcNAc_4$ and $GlcNAc_5$ (Usui *et al*. 1987). An endochitinase isolated from a *Bacillus* sp. was also able to perform enzymatic glycosylation using relevant glycosyl acceptors and donors under non-aqueous reaction conditions which permitted transglycosylation (Ochiai *et al.* 2004). The enzyme was shown to identify the oxazolinium compound as a transition state substrate therefore characterising it as an activated glycosyl donor

1.2.3 Chitosanase (EC 3.2.1.132)

Chitosanase or hexosaminidase activity has been detected in a variety of prokaryotes, fungi, plants as well as viruses (Yamada *et al.* 1997), where the enzyme catalyzes the hydrolysis of partially *N*-acetylated chitosan but not chitin. Chitosanases are classified into three classes according to their cleavage specificity: class I, enzymes that can split GlcNAc–GlcN in addition to GlcN–GlcN (Fukamizo *et al.* 1994), class II, enzymes that can only split GlcN–GlcN residues (Izume *et al.* 1992), and class III, enzymes that are able to split GlcN–GlcNAc in addition to GlcN–GlcN residues.

Akiyama *et al.* (1999) reported that prokaryotic chitosanases possess a conserved N-terminal module of 50 residues, and that two amino acids (Glu^{22} and Asp^{40} in *Streptomyces* sp. N174. chitosanase) were directly involved in the catalytic centre of the enzyme (Akiyama *et al.* 1999). The proposed mode of action showed a single displacement mechanism (Marcotte *et al.* 1996) with the postulated distance between the two catalytic carboxyl groups being ~13.8 Å. This indicates compatibility with an inverting mechanism for chitosan hydrolysis (Fukamizo and Brzezinski 1997); (Marcotte *et al.* 1996).

Amino acid sequence analysis of a *Paenibacillus ehimensis* showed a high degree of similarity to the chitosanase enzyme produced by *B. circulans* (86% similarity)

(Akiyama *et al.* 1999). Further enzyme characterisation found that the purified chitosanase enzyme from *P. ehimensis* had an approximate molecular weight of 31 000 daltons.

Structure and enzymology of chitosanases

Chitosanases are enzymes that catalyze the hydrolysis of chitosan, but not chitin. They are defined as carbohydrolases that hydrolyze partially *N*-acetylated chitosan, splitting the β-1,4-glycosidic linkages except the GlcNAc homo-linkages (Fukamizo *et al.* 1995) (Figure 9).

From the view point of sequence and folding similarities, chitosanases belong to families 46, 75 or 80 in the classification of GH enzymes (Henrisatt and Bairoch., 1996). Chitosanases of families 46 and 80 are enzymes from bacteria and several Chlorella viruses that share a conserved active site-related module (Tremblay *et al.* 2000).

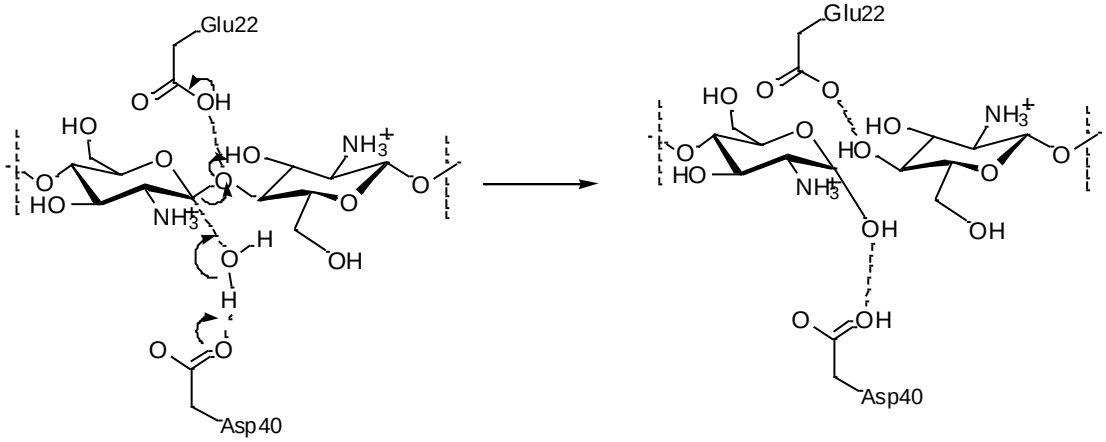


Figure 9. Biocatalytic mechanism of chitosanase action in *Streptomyces* sp. N174

Amino acid residues Glu^{22} and Asp^{40} were found to be critical for activity of the chitosanase enzyme in *Streptomyces* sp. N174. Glu^{22} functions as a proton donor while the residue Asp^{40} serves to activate a water molecule that attacks the anomeric carbon of the sugar residue.

Trembley *et al.* (2000) reported that most chitosanases studied belong to family 46 of the GH group of enzymes, however family 75 chitosanases includes those from three

fungal cultures, whereas family 80 chitosanases include those from the bacterium *Matsuebacter chitosanotabidus* as well as *Sphingobacterium multivorum*

Data obtained from crystallography and site-directed mutagenesis studies done on *Streptomyces* sp. N174 concluded that the Glu^{22} residue acts as a proton donor while the Asp^{40} residue is responsible for the activation of a water molecule which then attacks the C-1 carbon of the sugar residue located at the catalytic site (Boucher *et al.* 1995). On the basis of structure recognition sites, six binding subsites were identified from work done with each subsite being capable of accommodating one D-glucosamine residue (estimation using energy minimization studies). The involved amino acid residues includes Arg^{120} and Glu^{60} (subsite A), Pro^{152} (subsite B), Asp^{57} and Gly^{50} (subsite C), Ile^{49} and Glu^{197} (subsite D), Tyr^{34} (subsite E) and finally Asp^{201} (subsite F) (Fukamizo and Brzezinski 1997).

1.2.4 Hexosaminidase (EC 3.2.1.52)

Hexosaminidase enzymes catalyses the hydrolysis of terminal non-reducing *N*-acetyl-D-hexosamine residues in *N*-acetyl-D-hexosaminides. Based on sequence similarity studies done, hexosaminidases have been classified as belonging to family 20 of the GHs (Henrissat 1991). One of the conserved regions in the hexosaminidase enzyme produced by *Aspergillus wentii* A3 is centred on an aspartic acid residue which has been shown to be involved in the enzyme's catalytic ability (Bause and Legler 1980).

Humanization of therapeutic yeast generated proteins has seen the possible application of hexosaminidase enzymes together with a suite of other hydrolytic enzymes such as neuraminidase and galactosidases for use in removal of all terminal non-mannose residues, thus yielding a glycan structure which is more appropriate for human therapeutic use (Gerngross 2004).

1.3 Polysaccharide deacetylase enzymes

Chitin deacetylase enzymes (EC 3.5.1.41) (CDAH) belong to the polysaccharide deacetylase group of enzymes which are responsible for hydrolysing carbon-nitrogen

bonds other then peptide bonds. Other members of the polysaccharide deacetylase group include enzymes that hydrolyse 1- cyclic amides (EC 3.5.2.x), 2-linear amidines (EC 3.5.3.x), 3- cyclic amidines (EC 3.5.4.x) and 4- other substrates (EC 3.5.99.x). Typically CDAH enzymes act on linear amides with the substrate, chitin being hydrolysed to generate chitosan with acetate as a by-product of the reaction.

1.3.1 Chitin deacetylase-CDAH (EC 3.5.1.41)

Also known as chitin amidohydrolase, this enzyme hydrolyzes the *N*-acetamido groups present in GlcNAc residues in chitin (see Figure 10). In terms of mechanistic action chitin deacetylase enzymes convert chitin GlcNAc polymer into its deacetylated form (chitosan) which is a glucosamine polymer. Many fungal organisms such as *Mucor* spp., *Rhizopus* spp., *Colletotrichum* spp., as well as *Cunninghamella* spp., produce CDAH enzymes which may function to decrease the polysaccharide fungal cell wall susceptibility to chitinase as well as lysozyme enzymes which typically act of *N*-acetylated type substrates (Araki and Ito 1975). An increase in CDAH activity was noted in the fungal pathogen *Colletotrichum lindemuthianum* during fungal infection and initial growth in the host (Blair *et al.* 2006). The entomopathogenic fungi *Metarhizium anisopliae* upon germination on an isolated *Helicoverpa armigera* host was found to express both CDAH and chitinase enzymes which again may be responsible for altering the host cuticular chitin allowing for easier fungal penetration with a concomitant change in its own cell wall composition (Nahar *et al.* 2004). This would render the fungal pathogen less susceptible to the host insect's barrage of defence related enzyme/s.

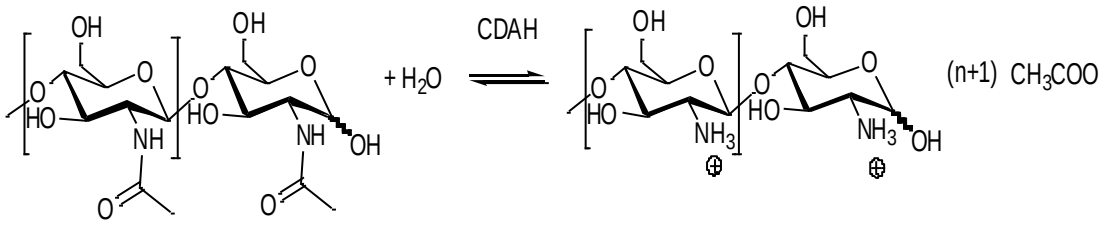


Figure 10. Chitin deacetylase activity (CDAH)

Chitin deacetylase results in non-specific deacetylation of chitin to yield chitosan with the concomitant release of acetate.

1.4 Uses and applications of chitinases

1.4.1 Agricultural application of chitinases

Chitinases have relevance in the agricultural fields since they may be employed in the biocontrol of plant pathogenic fungi and insects. Chitinases are also of interest due to their ability to generate chito-oligosaccharides which act as defence response elicitors in plants therefore signalling root nodule formation (Patil *et al.* 2000); (Jung *et al.* 2007). Chitosan oligomers have been implicated in inducing disease response genes such as endochitinase as well as endo-β-glucanase in many plant species (Hadwiger *et al.* 1994).

1.4.2 Allosamidin analogs for use as insecticidal agents

The structural as well as electronic properties of the oxazoline intermediate produced by family 18 chitinases during chitin hydrolysis can be used to attempt rational design of new active glycosidase inhibitors that are specific to family 18 chitinases (most insect chitinases belong to family 18 of the GH group of enzymes). Analogs of allosamidin with similar chair-like sugar conformation and containing the required positive delocalized charge could lead to the development of more effective chitinase inhibitors for employment in insect control (Brameld *et al.* 1998).

1.4.3 Application of chito-oligosaccharides

Chito-oligosaccharides produced by hydrolysis of chitin are of interest due to their potential biological activity. Homologous derivatives of chitopentaose as well as chitohexaose were found to demonstrate biological activity against tumor cells (Hinou *et al.* 2000). Both CHP and CHH compounds when tested *in vitro* showed inhibition of benzoate hydroxylation to salicylate in the presence of hydrogen peroxide with the two chito-oligosaccharides showing higher potency then known reference compounds (aminoguanidine, pyridoxamine and Trolox) (Chen *et al*. 2003). Substrates such as N, N^{I} diacetylchitobiose have also been used in the synthesis of novel disaccharide derivatives such as 2-acetamido-2-deoxy-*D*-allopyranose moieties. The monomeric chito-saccharide GlcNAc which is produced in the body by the incorporation of glucose into

glycoproteins and glycosaminoglycans also demonstrates anti-inflammatory action *in vivo* (Friedman and Skehan 2003).

1.4.4 Chitinase saccharide modification by transglycosylation

The transglycosylation potential of chitinases can be exploited to generate chito-saccharides of a desired length with modified glycosidic linkages and be used in the production of novel glycopeptides. Chitinases specifically show potential for the generation of heteropolysaccharides such as hyaluronan as well as chondroitin, but are not limited only to the production of anti-inflammatory drugs. The antioxidant properties of some chito-saccharides provide avenues into nutraceutical product formation that could find value in industry.

1.5 Uses and application of other GH enzymes

Hexosaminidase (EC 3.2.1.96) has been demonstrated to have both hydrolytic as well as transglycosylation activity with the acetamido group being identified as being crucial for enzyme activity (Fialova *et al.* 2004). Hexosaminidases were found to be able to catalyse N-acyl modified substrates with a demonstrated tolerance to sterical changes at the C-2 position.

The transglycosylation activities of chitin degrading enzymes have been studied for application in humanization of therapeutic proteins. Yeast and fungal cells are commonly applied to the production of many relevant therapeutic enzymes, however their applicability in this regard is limited due to the inherent problem of protein modification by mannosylation (Gerngross 2004). Yeast cell proteins are documented to be of a high mannose content which results in a short *in vivo* life of the enzyme as well as a loss in effectivity of the enzyme or possible immunogenic effect on the recipient (mannosylated proteins have a high affinity for mannose receptors in macrophages as well as endothelial cells).

1.6 Uses and applications of chitin-deacetylase and chitosan

Chitosan, which is the product of the chitin deacetylase enzyme, is the deacetylated form of chitin. Chitosan is used in photographic development procedures and in cosmetic preparations as an antifungal agent; chitosan has structural properties similar to glycosamino glycans and can therefore be considered for developing substratum for skin replacements. Due to the antifungal properties associated with chitosan, it has been investigated to determine its suitability for preparation of wound dressing material. Substrates containing GlcNAc were shown to promote growth of bifidobacteria in the gut therefore eliminating the establishment of intestinal pathogenic bacteria. Chitosan can be used for making contact lenses since it has good clarity, mechanical stability, gas permeability, fungistatic properties as well as immunological compatibility. The free amino and hydroxyl group in chitosan makes a useful chromatographic support which has been tested for separation of phenols, amino acids, nucleic acid and derivatives of inorganic ions. Lastly chitosan can be employed in bioremediation of metal and dye from wastewater systems and in the paper finishing process to add wet strength to the paper.

1.7 Potential applications of chitinolytic enzymes

Since chitinolytic enzymes function synergistically rather than individually it is likely that more than one enzyme is responsible for the observed activity. Considering the myriad of applications ranging from use in biological pest control to the application of de-acetylated chitin in the dye and textile industries, the potential for commercial application of the enzyme/s is very favourable.

1.8 Aim and objectives

The primary aim of the project is to assess the bio-catalytic potential of the chitinase produced by the environmentally isolated *P. chitinolyticus* strain. This assessment involves the quantification of chitinase activity as well as profiling of the enzyme (pH, temperature, substrate specificity screening and pI determination).

The environmental chitinolytic strain PBY will be evaluated by various gel electrophoretic methods to determine the number as well as types of chitin degrading enzymes being produced. Second dimensional separation of the chitinolytic enzyme/s will be performed to assess the nature of the chitinolytic isozyme/s present.

Enzyme specific assaying will be done to determine the nature (substrate preference) or conditions required for expression of the chitinolytic/ chitinase enzyme being produced by the studied strain. Baseline chitinase activity levels will be compared to levels obtained upon induction to ascertain whether the chitinase enzyme requires induction or is constitutively produced.

Chitinolytic enzyme purification will be attempted using chitin affinity chromatography as well as ion exchange chromatography, with the individual enzymes, once purified, being subjected to characterisation studies.

The kinetic properties of the enzyme was also be studied to ascertain the Michaelis-Menten constant (K_m) as well as the maximal velocity (V_{max}) values for the enzymes of interest being generated.

1.9 Thesis hypothesis

The study hypothesis is that the chitinase produced by an environmental isolate of *Paenibacillus chitinolyticus* would be biochemically different to those documented in literature.

CHAPTER TWO

GROWTH AND CHITINASE PRODUCTION BY AN ENVIRONMENTAL ISOLATE OF *P . CHITINOLYTICUS*

2 CHAPTER TWO - GROWTH AND CHITINASE PRODUCTION BY A NOVEL ENVIRONMENTAL ISOLATE OF *P . CHITINOLYTICUS*

2.1 Abstract

The biographical distribution of chitinases varies from aquatic to terrestrial environments. However the chitinase activity distribution in prokaryotic cell populations resides predominantly in *Aeromonas hydrophila* sp., *Bacillus* spp., *Serratia* spp., *Vibrio* spp., as well as *Streptomyces* spp. strains. Considering the vast industrial potential of *Bacillus* species (*Bacillus* spp.) as bio-catalysts isolation of chitinolytic microbes from these genera was considered favourable.

A simple *Bacillus* isolation strategy was tested and resulted in the purification and isolation of two novel environmental chitinolytic strains namely strain PBY and PBW. Chitinase purification as well as characterisation was done on strain PBY since the organism showed rapid chitin consumption on minimal media plates containing chitin (based on visible zones of clearing). An assumption was therefore made that the organism was either producing a more efficient chitinase enzyme or that the chitinase yields in strain PBY were better than strain PBW using the media and conditions tested. Sequence characterisation of strain PBY by 16s ribosomal deoxyribonucleic acid (rDNA) analysis revealed that the strain was most alike to a *P. chitinolyticus* strain. Induction on minimal media containing purified crab chitin resulted in sufficient chitinase activity after 72 hours of incubation at 25^{o}C. Chitinase enzyme purification was done using both a chitin affinity purification strategy followed by anion exchange chromatography (anion EX). The final chitinase purification yield was 7% with a fold purification of 106. This overall yield was significantly lower than that documented in *Vibrio carchariae* however this is most likely due to the purification conditions employed not having been optimised.

2.2 Introduction

In most organisms chitinase activity is controlled by a repressor-inducer system where chitin or some of its degradative products serve as chitinase inducers (Monreal and Reese 1969). Further studies have shown that chitinolytic organisms have trace quantities of constitutive enzymes that are expressed under conditions of starvation thus enabling the utilization of chitin as a carbon and nitrogen source.

2.2.1 Nitrogen source variations on chitinase activity induction

Nawani and Kapdnis found that the addition of yeast extract or ammonium sulphate in the chitinase induction medium used for *Streptomyces* spp. strains NK1057, NK528, NK951 resulted in an increase in chitinase activity (Nawani and Kapadnis 2005). These nitrogen sources were tested at levels ranging from 0.1 g/L to 0.4 g/L using a 2-level fractional factorial method for statistical assessment of combined variables on chitinase activity. An increase in chitinase activity was found to be due to the growth of the strains on the two nitrogen sources supplemented in the media. Yeast extract was also found to contain various nitrogenous compounds, growth factors and oligomers of GlcNAc which could have had a stimulatory effect on growth as well as induce chitinase activity production. Work done on *Paenibacillus* sp. D1 again verified the importance of yeast extract with a Plackett-Burman study revealing its central importance to induction of chitinase activity (Singh *et al.* 2009).

Chitinase enzyme production in *Serratia marcescens* was enhanced by the addition of 0.02% yeast extract (Monreal and Reese 1969) whereas high levels of chitinase activity were reported for *Serratia liquefaciens* when casamino acid, aspartic acid, tryptophan or tyrosine were added. In *Pyrococcus kodakarensis* chitinase activity was induced by the addition of 0.25% yeast extract and 2.5% casamino acid (Bhushan and Hoondal 1998), whereas induction in *Bacillus* strain MH-1 was observed after the addition of 0.05% yeast extract and 0.7% ammonium sulphate (Sakai *et al.* 1998). Finally chitinase induction studies done on *Vibrio harveyi* and *Vibrio carchariae* included the addition of 0.5% yeast extract and 0.5% bactopeptone or tryptone into the induction medium(Svitil *et al.* 1997); (Suginta *et al.* 2000).

2.2.2 Carbon source variations on chitinase activity

Chitin addition into the media in varied forms was found to induce chitinase activity in many strains. Chitinase production in *Microbispora* sp. V2 was improved by the addition of colloidal chitin instead of chitin flakes (Nawani *et al.* 2002), whereas *Serratia marcescens* induction studies showed an increase in activity yields when swollen and colloidal chitin was used instead of mushroom or beetle chitin (Monreal and Reese 1969). In *Pyrococcus kodakarensis*, *Bacillus* strain MH-1 as well as *Vibrio harveyi* chitinase induction involved the addition of 0.5% weight/volume (w/v) chitin, while *Bacillus circulans* WL-12 was induced using 0.2% (w/v) chitin (Bhushan and Hoondal 1998); (Svitil *et al.* 1997; Sakai *et al.* 1998).

In general glucose addition into media was found to repress chitinase synthesis in almost all microorganisms studied thus indicating that catabolite repression may be involved in the regulation of microbial chitinases (Saito *et al.* 1998). In *Streptomyces lividans* the glucose kinase (Glk A) was shown to play a role in chitinase repression.

2.2.3 General induction conditions

Cell culturing on minimal media was done at 25^{o}C for 3 days on an orbital shaker set at 200rpm. After culturing the cells were centrifuged and the extracellular fraction concentrated and processed by chitin affinity purification as well as anion EX chromatography to enable purification of the extracellular chitinase enzyme (the initial sample concentration was done by PEG dialysis using a 10kDa cut-off membrane and PEG 20 000).

2.2.4 Chitinase purification strategies

Enzyme purification strategies typically included chitin affinity adsorption (Bhushan and Hoondal 1998); (Watanabe *et al.* 1990; Svitil *et al.* 1997; Sakai *et al.* 1998; Suginta *et al.* 2000); some early fractionation by ammonium sulphate precipitation (50-80%, 40%) (Watanabe *et al.* 1990; Svitil *et al.* 1997; Bhushan and Hoondal 1998) and finally enzyme fractionation by anion EX chromatography (Sakai *et al.* 1998) or size exclusion chromatography (Svitil *et al.* 1997; Bhushan and Hoondal 1998) .

2.3 Aims

- To selectively isolate a chitinase producing strain from an environmental sample
- To induce strain PBY chitinase production in cells
- To ascertain whether chitinase activity is constitutive or inducible
- To complete validation of the enzyme specific chitinase assaying
- To purify the native chitinase enzyme using ion-exchange as well as chitin affinity separation techniques

2.4 Materials and Methods

2.4.1 Microbial isolation and culturing studies

Organism and media

Strain PBY was isolated by selective enrichment from an environmental soil sample obtained from a previous lab experiment. Taxonomic identification of the culture was done by16 S rDNA analysis (Molecular and Cell Biology Group, University of Cape Town). Note that taxonomic reclassification of the strain by Kuroshima (1996) resulted in the strain name being changed from *Bacillus chitinolyticus* to *P. chitinolyticus* (Kuroshima *et al.* 1996).

Isolation strategy

The procedure followed for enrichment of *Bacillus* spp. from environmental samples was as follows. Approximately 1 gram (g) of soil was added to 9 millilitres (mL) of nutrient broth (0.5% (w/v) peptone from meat, 0.3% (w/v) meat extract, 1% (w/v) agar and incubated overnight at 37 degrees celsius (^{o}C) with shaking. Another sample (equivalent in weight) was added to 9 mL of buffered peptone water (composition: 1% (w/v) peptone, 0.5% (w/v) sodium chloride (NaCl), 0.9% (w/v) disodium hydrogen phosphate dodecahydrate, 0.15% (w/v) potassium dihydrogen phosphate) and incubated at 45^{o}C for 30 minutes. The resulting solutions were then centrifuged at 11 000 x g or 15 minutes in a Pico Heraeus micro-centrifuge. After incubation the samples were removed and dehydrated by the addition of 50% volume/volume (v/v) ethanol, followed by an

incubation step at room temperature for 1 hour. The supernatant containing ethanol was decanted and the remaining pellet incubated at 105^{o}C for 5 minutes to facilitate removal of any residual ethanol. Finally the dried pellet was reconstituted in 20 mL of sterile distilled water and serial dilutions of each sample were made and plated onto nutrient agar plates supplemented with polymyxin B. The plates were then incubated at 37^{o}C and isolation of the single colonies was done. The purified colonies were grown on chitin containing plates comprising 0.5% (w/v) crab shell chitin made up in 100 mM potassium di-hydrogen orthophosphate buffer, pH 7.0, to identify any potential chitinolytic strains.

Culturing and enumeration

Cell culturing was done using Tryptone Soy Broth (TSB) obtained from Anatech (Scharlau Chemie, S.A. Barcelona). The media comprises of the following: 1.7% (w/v) casein peptone, 0.3% (w/v) soya peptone, 0.5% (w/v) NaCl, 0.25% (w/v) dipotassium phosphate, and 0.25% (w/v) dextrose (media pH is approximately 7.0). The media used for induction of chitinase activity comprised of 1.0% (w/v) yeast extract and 0.5% (w/v) purified shrimp/ crab chitin (Sigma-Aldrich) and 0.05% (w/v) calcium carbonate dihydrate made up to volume using 100 mM potassium di-hydrogen orthophosphate buffer, pH 7.0.

Routine maintenance of strain PBY

Strain PBY cells were inoculated onto minimal media plates as well as TSB agar plates. Colonies from a 48 hour old plate were used to inoculate TSB flasks. These flasks were incubated at 25^{o}C on an orbital shaker set at 220 revolutions per minute (rpm) for 24 to 48 hours prior to use of the biomass for cryo-preservation. The spent supernatant was removed and the biomass re-suspended in sterile phosphate buffer containing 15% (v/v) glycerol. The cryovial contents were then stored at -75^{o}C.

Morphological assessment of strain PBY

Routine sub-culturing of cells on plates were done with the resulting cultured plates being stored at 4^{o}C. Cells cultured on both rich (TSB) as well as minimal media were introduced onto microscope slides with accompanying cover slips and viewed using the Olympus BX40 phase contrast microscope. Images were acquired using the 100 x objective under oil immersion.

Growth rate of strain PBY on minimal media

Cells from a 48 to 72 hour old plate were used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of TSB. Flasks were inoculated overnight and the cultured cells used as an inoculum train for sub-culturing again onto TSB (250 mL flask containing 50 mL TSB broth). Growth was monitored by spectroscopy with readings taken at 600 nm as well as 660 nm to determine the relative biomass in suspension. Once the cell concentration in the flask had reached an optical density (OD) of 1.0 at 600 nm wavelength the flasks were removed from the shaker and used to inoculate either TSB or minimal media flasks (1000 mL Erlenmeyer flask containing 200 mL broth). Samples were removed hourly from duplicate TSB flasks and readings taken using a Beckman Dupont DU 800 Spectrophotometer.

2.4.2 Chitinase activity Assessment

Assay validations

A commercially available chitinase produced by *S. griseus* and purchased from Sigma-Aldrich was used as a control for validation of assay conditions with 4-methylumbelliferyl chitobioside (4-MU CHB) used as substrate. The presence of β-N acetylglucosaminidase activity was investigated using a Jack bean β-*N*-acetylglucosaminidase with *N*-acetylglucosaminide used as substrate (both purchased from Sigma-Aldrich).

Substrates used for activity assessment

Substrates such as GlcNAc; CHB; N, N^{I}, N^{II} triacetylchitotriose (CHT); CHP as well as the fluorescent substrates and controls used for assaying activity were purchased from Sigma-Aldrich. These included 4-methylumbelliferone (4-MU), 4-methylumbelliferyl glucosaminide (4-MU Glc), 4-MU CHB and finally 4-methylumbelliferyl chitotrioside (4-MU CHT). All the substrates were solubilised in low volumes of dimethylformamide (DMF) before making up to the required volume using deionised water. A primary 100 µM stock solution was prepared and stored in a dark container at 4^{o}C to prevent photodecomposition of the substrate. Successive dilutions of the primary stocks of 4-MU GlcNAc, 4-MU CHB, as well as 4-MU CHT generated the varied substrate concentrations (13.8-122 nM range).

Assay conditions

Enzyme activities and kinetic data were calculated from data gathered using 4-methylumbelliferyl substrate analogues such as 4-MU Glc, 4-MU CHB and 4-MU CHT. The amount of 4-MU released was measured using an LS-55 Perkin Elmer Fluorescence Spectrophotometer with the excitation wavelength set at 360 nm and emission wavelength readings taken at 440 nm. Slit widths of 10 nm were used and the scan speed was set at 400 nm/ second. With respect to data collection per study, triplicate independent kinetic readings were taken and the data averaged to ascertain the standard deviation (*S*) of the data.

A 4-MU standard curve was used for extrapolating unit activity from the fluorescence data obtained. The 4-MU standard curve concentrations ranged from 13.88 nM to 222 nM, with triplicate readings averaged to ascertain the mid-point value.

4-MU standard curve for activity quantification

The substrate 4-MU was found to be insoluble in water and was dissolved in a small volume of methanol prior to being made up to the desired volume with 100 mM phosphate buffer pH 7.0. Concentrations of 4-MU used in the standard curve ranged between 12.5 nM and 200 nM with triplicate readings taken to ascertain data accuracy.

Standard chitinase reactions

All reaction assays were done using a 100 mM potassium di-hydrogen orthophosphate buffer at pH 7.0. Substrate concentrations were standardised at 10 µM for all kinetic data obtained. Kinetic readings were commenced without enzyme addition to obtain blank values. Enzyme addition (29 µg protein per mL reaction volume) was then followed by reaction incubation at 25^{o}C temperature with intermittent mixing between minute readings. Reaction rates were calculated from kinetic data gathered over a 5 to 10 minute incubation period with triplicate readings averaged to calculate unit activity values.

Graphs were constructed using the kinetic data obtained by fluorescence spectroscopy. The data points from commencement till the last reading were used to plot a curve of the

raw data which had a linear curve drawn for each set of data points. The equation of the curve was then used to calculate the reaction rates (not the raw data). The reaction rate was therefore calculated as half of the total change in absorbance divided by the time it took for this change to occur (see Appendix one, Figure 36 for an example of the reaction rate calculation used).

2.4.3 Chitinase purification

Processing and purification of the extracellular protein fraction from strain PBY

Cells of strain PBY were cultured at 25^{o}C in 5 x 500 mL Erlenmeyer flasks containing 100 mL minimal media for 72 hours with continuous agitation at 220 rpm. Cells on harvesting were centrifuged at 11 000 x g for 20 minutes at 4^{o}C with the pellet being discarded and the supernatant used for enzyme purification.

The cell free extract (CFE) obtained after culturing was concentrated by dialysis with polyethylene glycol (PEG) 20 000 using a 3.5 or 10 kDa limit pleated dialysis tubing until the solution was a third of the original volume (10 kDa dialysis tubing was preferentially used although a 3.5 kDa membrane was used when no 10 kDa membrane was available). The concentrated extract was then subjected to chitin affinity purification (5 mg chitin per mg protein) with the enzyme substrate complex being incubated at 4^{o}C for an hour. After a water wash step to remove any unbound contaminating proteins, the enzyme was eluted from the substrate by incubation at 25-30^{o}C overnight with agitation. The enzyme post affinity purification was subjected to anion EX chromatography to further resolve the chitinolytic enzymes in the sample.

The concentrated supernatant (minus salts and small peptides) was buffer exchanged overnight against 20 mM tris-2-amino-2-(hydroxymethyl)-1,3, propandiol (Tris), pH 9.0 for all anion EX studies or dialysed against 20 mM morpholinoethanesulfonic acid (MES), pH 6.0 for all cation exchange chromatography studies (cation EX) (100 x buffer volume exchange). The buffer exchanged samples were once again concentrated against PEG 20 000 for volume reduction prior to use in ion-exchange purification studies (see Figure 11).

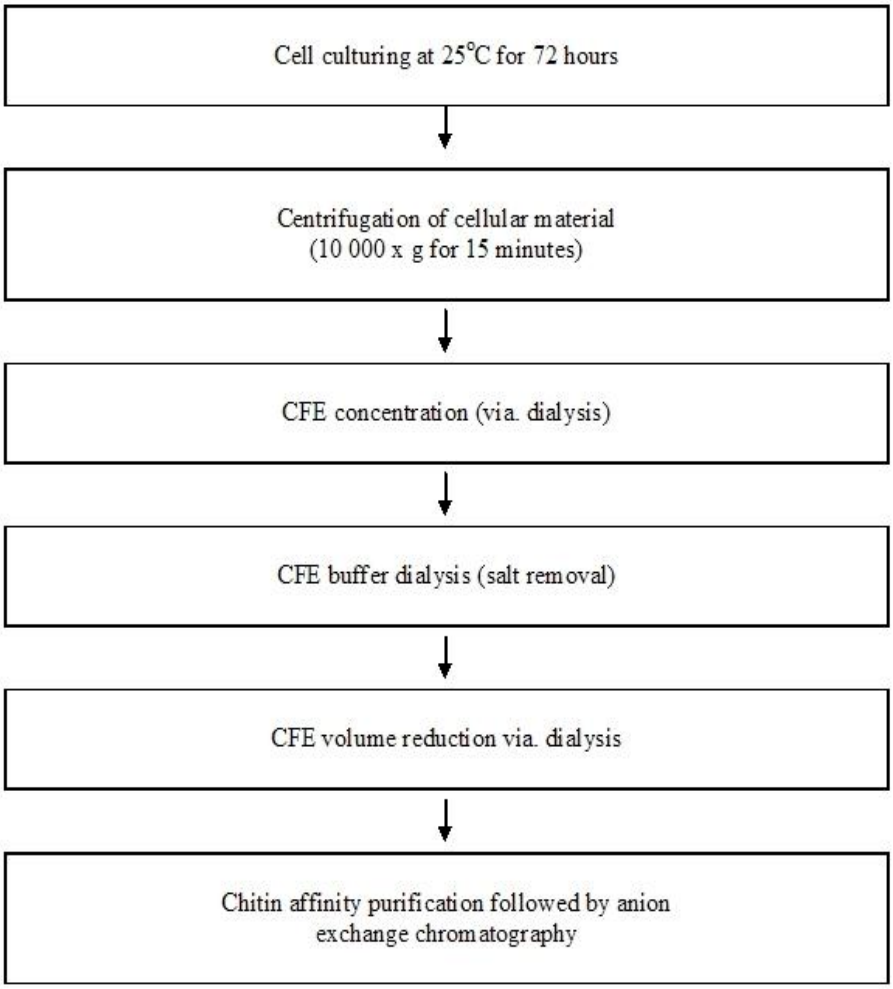


Figure 11. Purification strategy used for chitinase isolation

Preparation of columns for chitinase purification

The commercially available purification columns were washed with 5-10 mL of the start buffer at a flow rate of 1 mL/min (20 mM Tris, pH 9.0 or 20 mM MES, pH 6.0) to remove any preservatives from the column and to allow for column equilibration. The enzyme sample that had previously been buffer dialysed and concentrated was injected onto the column and the residual unbound proteins/ salts were washed off by applying 5 mL of the start buffer. Sample elution from the column was commenced by application of 5 to 10 mL of elution buffer (20 mM Tris–pH, 9.0 with 1 M NaCl or 20 mM MES, pH 6.0 with 1 M NaCl), with 5 mL protein fractions collected and analysed for protein quantification purposes as well as chitinase activity.

Resins tested for protein separation were purchased as 1 mL pre–packed columns with set column conditions for use when using the AKTA purification system. These include cation EX resins (HiTrap SPFF, HiTrap CMFF and HiTrap SPXL), as well as anion EX resins (HiTrap DEAE FF, HiTrap Q FF, HiTrap QXL and HiTrap ANX FF) purchased from GE Healthcare.

For large scale chitinase purification a 25 mL QFF Pre-packed column was used. The partially purified chitinase sample after chitin-affinity purification was applied onto the column and the residual unbound proteins washed off using a 20 mM Tris buffer (pH 9.0). Sample elution from the column was done using a 1M NaCl buffer solution, with 10 mL fractions collected and assessed using 10 µM 4-MU CHB as substrate

Chitin affinity purification

The extracellular protein after concentration and diafiltration was subjected to substrate affinity purification using pure crab chitin from Sigma-Aldrich (5 mg chitin/ mg protein). The enzyme-substrate complex was incubated at 4^{o}C with gentle agitation for 24 hours to allow for binding of the enzyme to the substrate. After centrifugation at 11 000 x g for 20 minutes the supernatant post binding was discarded and the pellet water washed to remove any unbound contaminating proteins prior to the chitinase enzyme being eluted by incubation at 30^{o}C (48 hours, 90 rpm on an orbital shaker). The sample was centrifuged upon clearing and the pellet discarded (11 000 x g, 20 minutes). Diafiltration was done using a 100 mM Tris buffer (pH 7.0) to remove any low molecular weight chito-oligosaccharides in the sample prior to volume reduction using a 3.5 kDa snakeskin dialysis tubing using PEG 20 000.

Protein quantification

The total protein content in samples requiring analysis was assayed using the Bio-Rad reagent which is based on the Bradford method for quantifying protein concentration in samples. Bovine Serum Albumin (BSA) was used as the protein standard. The BSA solution absorbances were measured at 595 nm on a Beckman Coulter DU800 spectrophotometer. The absorbance data obtained was used to generate a BSA standard

curve and the linear equation of the curve was used to determine protein concentrations of all unknown samples (Bradford 1976).

Readings were taken in triplicate to ascertain reproducibility as well as accuracy of data. The samples were mixed by inversion and incubated at 25^{o}C for 5 minutes prior to readings being taken. Experimental samples were diluted when needed and studied in the same manner.

2.5 Results and Discussion

2.5.1 Isolation and growth conditions used for strain PBY

The environmental strain PBY used was obtained by selective enrichment of an environmental sample in the presence of chitin. A *Bacillus* enrichment protocol was employed for the selective isolation of *Bacillus* strains with two bacterial strains showing chitinase activity. A decision was taken to study the strain that showed more rapid chitin consumption on minimal media plates (5% purified chitin, 1.5% agarose made up in phosphate buffer, pH 7.0).

The selected strain was submitted for 16S rDNA sequencing to determine identity. The study indicated that the culture was most alike to the *P. chitinolyticus* (>99% identity). Taxonomic studies done using 16S rDNA sequence data as well as DNA–DNA hybridization studies resulted in the reclassification of *Bacillus chitinolyticus* to the genus *Paenibacillus* (Lee *et al.* 2004).

The purified culture demonstrates morphological similarities with documented information on a type strain *P. chitinolyticus* (NRRL B-23119). Previous characterisation work done on *P. chitinolyticus* cells suggest the cells are rod-shaped, motile (by peritrichous flagella) gram-positive prokaryotes with evidence of chitin decomposition (Kuroshima *et al.* 1996). This was observed with the environmental strain characterised in this study (Figure 12).

Cells of strain PBY when cultured on minimal media with chitinase induction on pure crab shell chitin showed visibly different morphological growth properties in comparison to colony characteristics on rich media (TSB/TSA). The same cells when induced for chitinase activity produced brighter yellow colonies with feathering formation at the colony edges which could be viewed as an indicator for successful chitinase induction (Figure 13). Chitinase activities using fluorescent substrates confirmed the presence of chitinase in the extra-cellular fraction.

In terms of storage conditions, cell freezing with glycerol at -75^{o}C worked well with cell viability maintained even after 6 months of storage under these conditions. Working cell banks of the culture also underwent serial transfer to defined media plates as well as TSB agar plates and was kept at 4^{o}C for inoculation purposes.

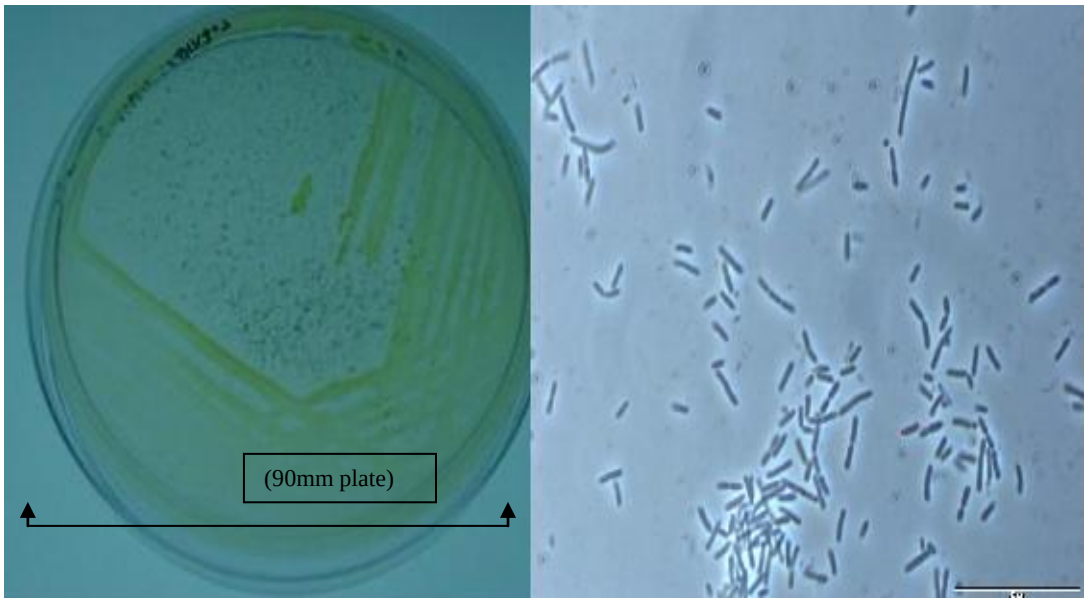


Figure 12. Physical growth of strain PBY on induction media as well as morphological characteristics of cells

Left image: Induced cells colonies were found to be bright yellow in colour. Right image: Microscopic images of strain PBY showing typical rod-shaped cell morphology. Cells were inoculated on media comprising 1% yeast extract and 0.5% purified crab chitin (Sigma-Aldrich) made up to volume in a 100 mM potassium–dihydrogen orthophosphate media. Image taken using a Sony 3CCD colour video camera/ CCD iris attached to an Olympus BX40 light microscope. Images acquired using the 100 x magnification objective with sample viewing under oil immersion.

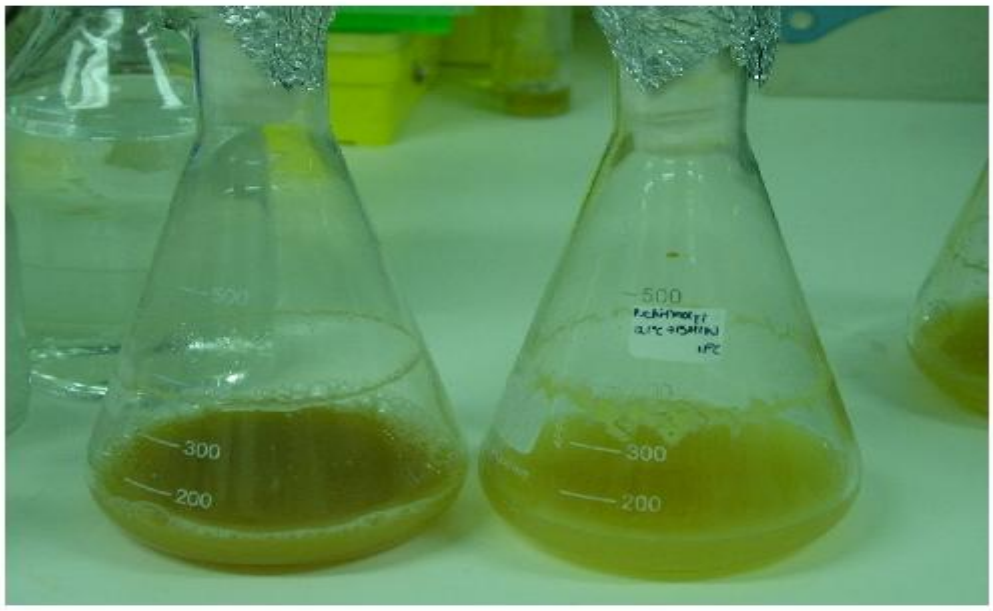


Figure 13. Appearance of flasks containing induced (right) and non-induced (left) strain PBY cells

Left: Flask of strain PBY cells grown on TSB where the cells were not induced; right: 48 hour flask of strain PBY with cells grown in minimal media containing chitin. Cells upon induction turn bright yellow thus providing a physical indication of successful induction.

Biomass determination on minimal media was not possible due to the presence of the crab shell chitin which co-sedimented after centrifugation and interfered with the actual biomass data obtained. However, the cell doubling/generation time during exponential growth on TSB was found to be 50-54 minutes at 25^{o}C (Figure 14) (see Appendix 2, Table 11 for optical density data used). Biomass cultivation overnight in TSB is therefore sufficient to enable the production of enough cellular material for transfer into the induction medium.

Cell size determination was done using an Olympus BX-40 light microscope, with image capturing done using an Olympus CMAD-3 camera. The mean cell size was calculated from an average of 10 individual cell measurements, with the Σ determined to be 4.26 μm (see Appendix 3, Table 12 for raw data). The SD of the data was 0.67, while the data variance was calculated to be 0.448.

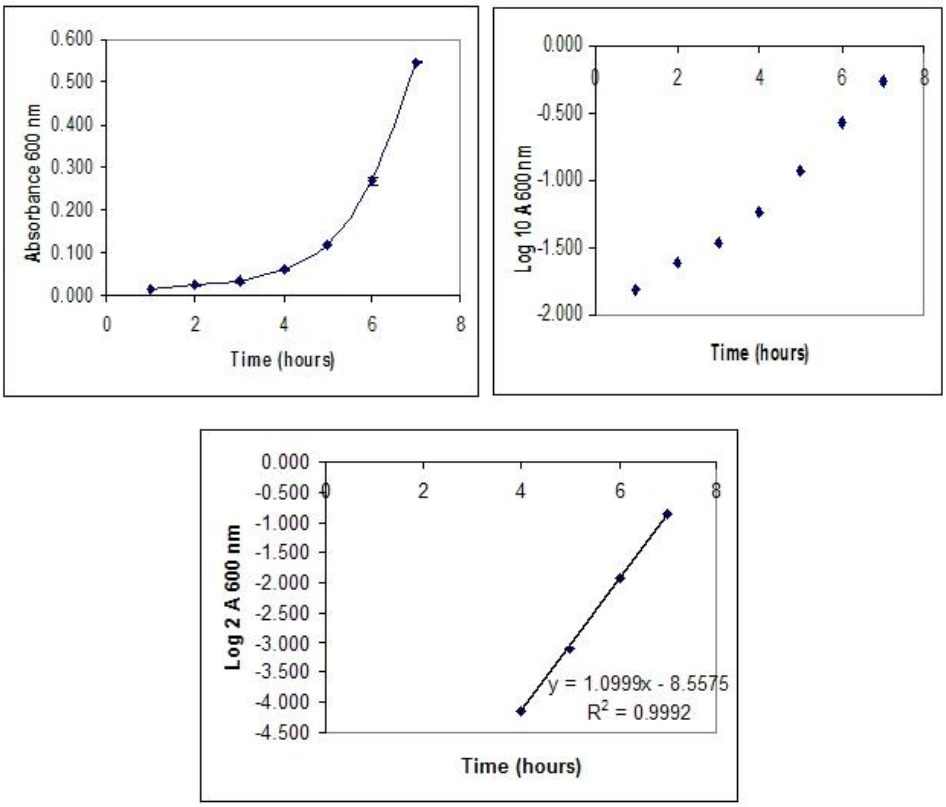


Figure 14. Strain *PBY* growth curve on tryptone soy broth**.**

Cell culturing was done on TSB with samples extracted hourly for spectrophotometric optical density assessment at 600 nm. Cells were cultured using a common inoculum source (overnight incubation of cells from TSB agar plate into TSB with flasks incubated at 25^{o}C with shaking) with 1 mL of the actively growing cell suspension used for inoculating 200 mL of TSB (3 x 1 L Erlenmeyer flasks). After inoculation the flasks were incubated at 25^{o}C with shaking on an orbital shaker set at 200 rpm. Values from duplicate flasks were averaged and the SD values were calculated for each data point.

A: Growth curve on TSB;

B: Log $_{10}$ of absorbances at 600 nm;

C: Log $_{2}$ of absorbance values that appear to be in logarithmic phase of growth (4 hours-7 hours).

Cells from strain PBY after culturing on TSB were centrifuged and transferred into the induction media containing pure chitin (200 mL of an overnight grown cell suspension was centrifuged and the cells transferred into 100 mL of the induction medium). When cultured in a defined media, cells showed significant chitinase activity after incubation at 25^{o}C for 72 hours with shaking (Figure 15) (see Appendix 4, Table 13 for the raw data used and Table 14 for statistical analysis of the raw data). The overall chitinase activity levels (U/mL) increased five fold from day one to day two and was sixteen fold higher by

day three. Activity levels were sufficient at this point to enable further enzyme purification and characterisation studies to be done.

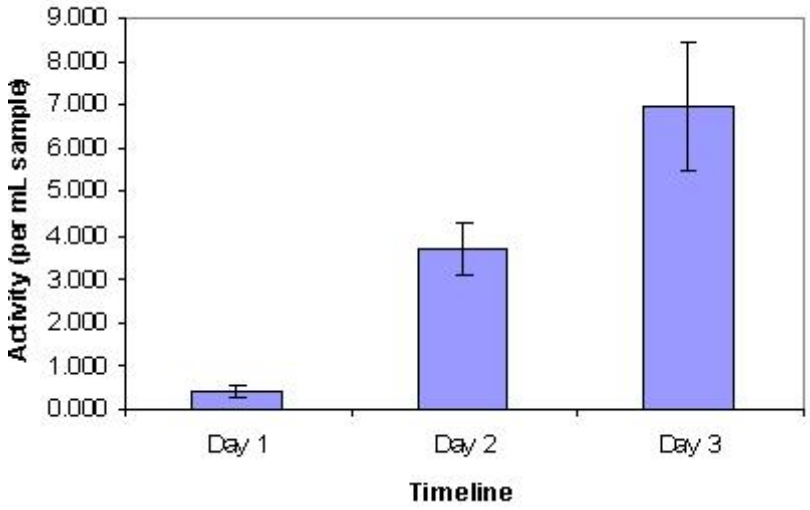


Figure 15. Chitinase enzyme expression on minimal media over 72 hours

Strain PBY cells were grown on minimal medium for a period of three days at 25^{o}C with continuous agitation at 200 rpm on an orbital shaker. Activity levels were assessed using 4-MU CHB with fluorescence emission readings taken at 440 nm and excitation set at 360 nm. Slit widths of 10 mm were used and kinetic readings were assessed over 5 to 10 minutes. Chitinase activity was expressed as the release of 1 µmole 4-MU per minute at 25^{o}C temperature.

4-MU standard curve

A 4-methylumbelliferyl standard curve was prepared to quantify chitinase activity by extrapolating reaction rate data from fluorescence data gathered. Results obtained from triplicate samples showed good reproducibility for 4-MU concentrations ranging between 13.88 nM–220 nM which was within the linear range of the fluorescence spectrophotometer. Data obtained showed a graph with a regression of 0.9981 (Figure 16). The SD values calculated for the individual points ranged from 2.67 to 16.87 (see Appendix 5, Table 15 for the fluorescence data used in constructing the 4-MU standard curve).

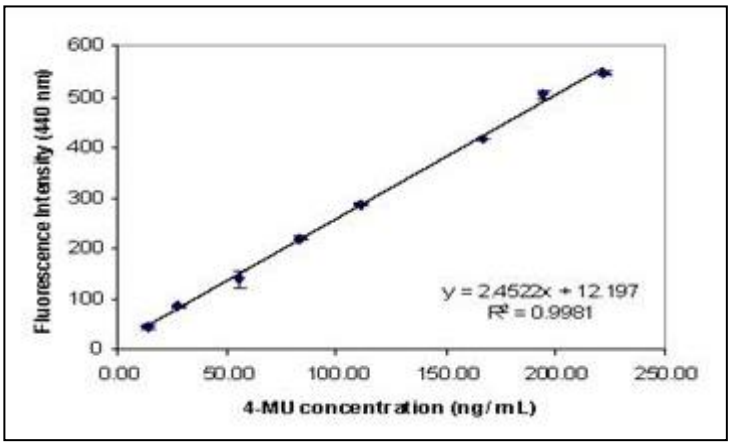


Figure 16. A representation of a 4-MU calibration curve

4-MU Calibration curve data was obtained using triplicate sample results (13.88 nM to 220 nM 4-MU). Readings were taken at 25^{o}C using an LS 55 fluorescence spectrophotometer from Perkin Elmer with the excitation wavelength set at 360 nm and emission data gathered at 440 nm. Slit widths were set at 10 nm and the scan speed was 400 nm/second.

2.5.2 Chitinase assay validation using *Streptomyces griseus* chitinase

Chitinase assay validation was done using a commercially available *S. griseus* chitinase purchased from Sigma-Aldrich as well as 4-MU CHB as substrate. The slope of the curve was used to determine the unit/ mL activity of the enzyme tested with unit activities ranging from 129 mU to 17.9 mU/ mL (Figure 17). The effect of the enzyme concentration on chitinase activity was also tested (Figure 18).

Control assays were run on the buffer alone as well as the buffer containing DMF and 10 µM 4-MU CHB in combination. This was done to ensure the absence of interference from any of the assay reagents used (Figure 19).

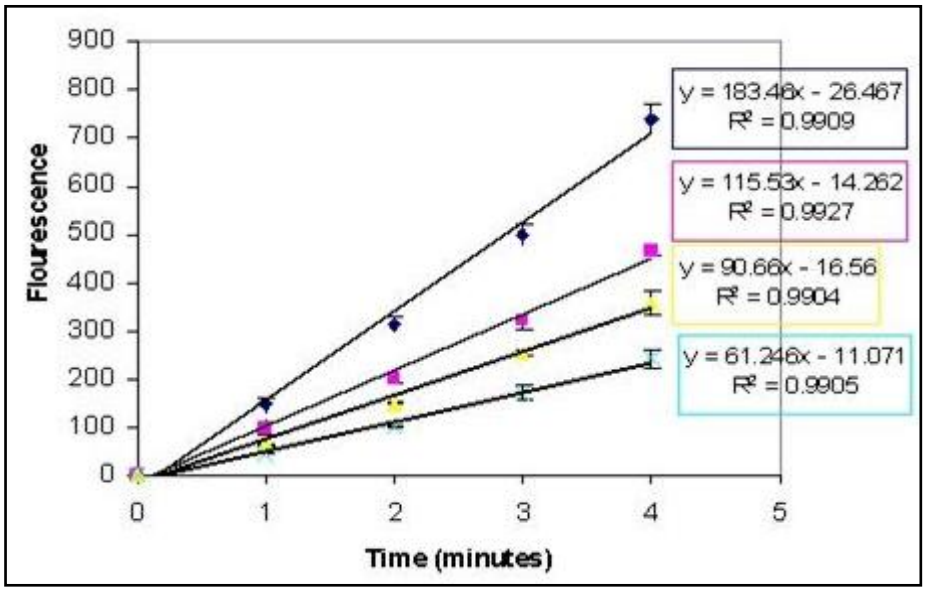


Figure 17. Validation of chitinase assay conditions using *S. griseus* chitinase (Sigma-Aldrich)

The effect of time on enzyme activity using a series of subsequent enzyme dilutions. The enzyme concentrations used are indicated as follows: dark blue – 64.8 mU; pink – 39.4 mU; yellow – 28.8 mU; cyan – 17.9 mU. Kinetic assaying was done using 4-MU CHB as substrate with triplicate readings over a five minute period averaged to determine the SD of the data. The excitation wavelength was set at 360 nm while the emission wavelength was 440 nm. Slit widths were set at 10 nm with the scan speed being 400 nm/second.

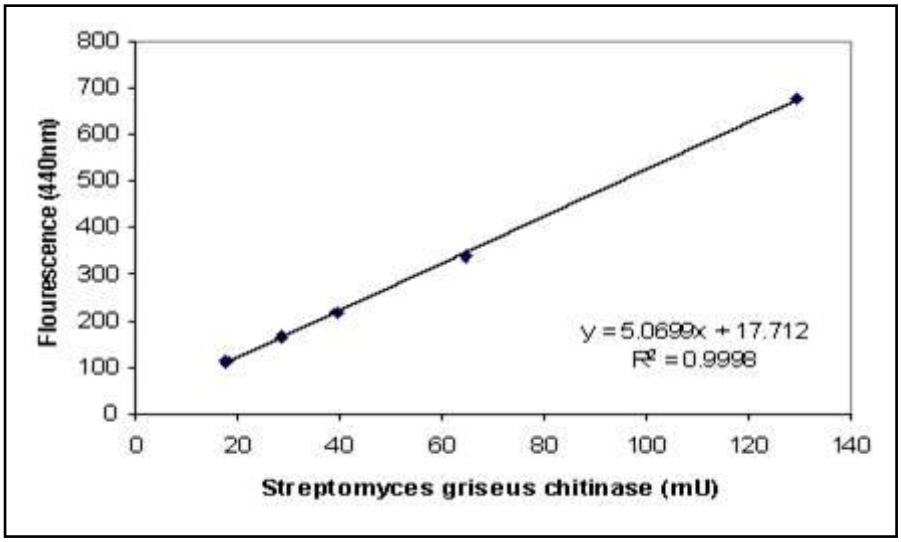


Figure 18. The effect of varied *S. griseus* chitinase concentrations on chitinase activity

Initial velocities obtained by interpolation of curves in Figure 17.

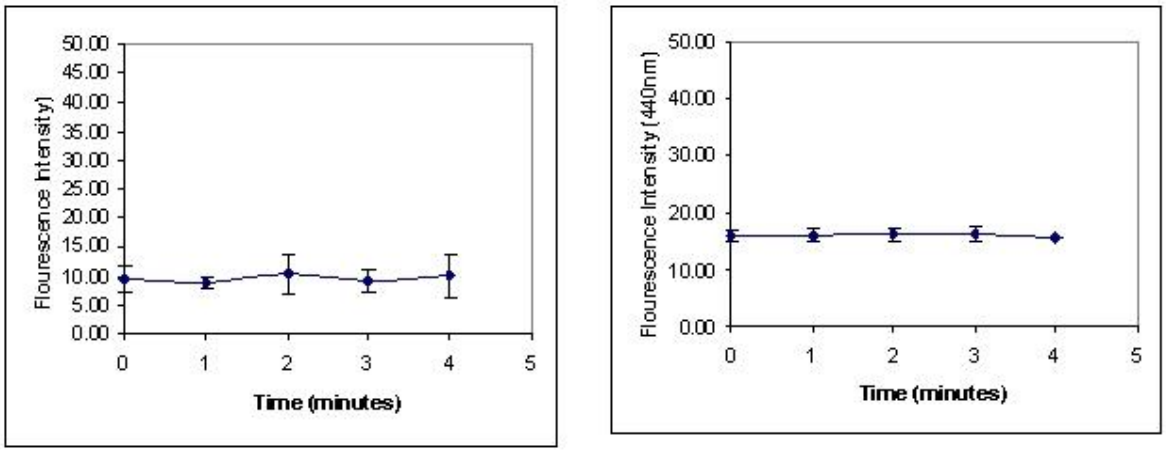


Figure 19. Representation of a typical buffer and substrate control sample when using 4-MU CHB as substrate

Left: Buffer control containing 100 mM phosphate buffer, pH 7.0 only; Right: Buffer containing 10 µM 4-MU CHB which was initially solubilised in DMF. Readings were taken at minute time intervals over a four minute assaying period. The excitation wavelength was set at 360 nm while emission spectral data was taken at 440 nm. Slit width were set at 10 nm while the scan speed was 400 nm/sec.

2.5.3 Hexosaminidase activity assay validation using Jack Bean hexosaminidase

Assaying for hexosaminidase activity was done to determine whether the extracellular fraction from strain PBY could hydrolyse terminal non-reducing sugar residues. Assay

optimization was done using Jack Bean hexosaminidase (Sigma-Aldrich) with activity detected against 4-MU Glc. There was however no detectable activity from the crude and purified enzyme sample against this substrate, thus indicating the absence of hexosaminidase activity in strain PBY.

2.5.4 Chitinase purification using chitin affinity purification

Affinity binding using purified chitin initially showed little activity loss in solution since the binding had been carried out using non acid-swollen chitin. The use of acid-swollen chitin in which most of the calcium in the chitin is precipitated by acid hydrolysis and the substrate hydrolysed into smaller chito-oligosaccharides is likely to result in better enzyme binding. However 24 hours after commencing with the enzyme-substrate binding process over half of the activity initially observed in solution at 1.5 hours had disappeared (104 U chitinase in solution at 24 hours incubation versus 262 U chitinase activity at 1.5 hours)(Figure 20). A single data point was assayed for the time 0 sample in Figure 20, thus no SD is available for this point.

The chitinase activity observed in solution even after a prolonged substrate binding period could be due to the use of insufficient chitin during the enzyme-substrate binding process. Alternatively the presence of salts in the sample after diafiltration could interfere in the effective binding of the enzyme to the substrate. After completion of the binding process little chitinase activity was detected in the supernatant and no activity was found in the following water wash sample. The documented negative activity data shown for the water wash sample is due to the reaction rate data obtained being low and falling outside of the linearity of the 4-MU CHB assay used (Figure 22).

The chitinase elution profile showed a three-fold increase in chitinase activity in solution between 24-48 hours after elution had commenced (88 U chitinase activity at 24 hours versus 233 U at 48 hours) (Figure 21). After substrate binding most of the chitinase activity lost during elution from the substrate is likely to have remained bound to the chitin after centrifugation. It is possible that an increase in the enzyme elution time allocated could have resulted in an increase in the percentage of recovered chitinase. It is

also possible that some activity loss may have been incurred as a result of the enzyme incubation at 30^{o}C for 48 hours.

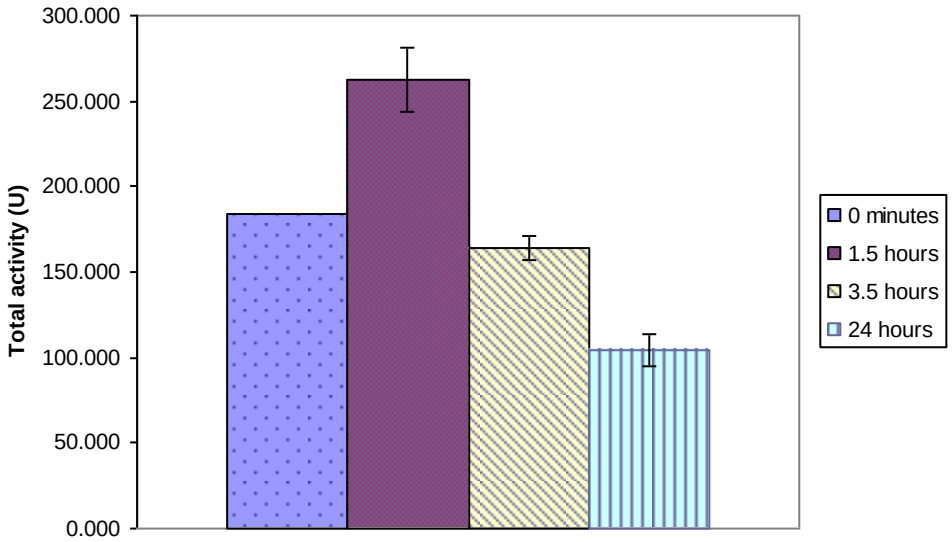


Figure 20. Chitinase binding during substrate affinity purification

Enzyme binding was done using 5 mg purified crab chitin per mg protein. The enzyme substrate mixture was incubated at 4^{o}C with gentle agitation with samples taken at 0 minutes, 1.5 hours, 3.5 hours as well as 24 hours. The samples were assayed using 10 µM 4-MU CHB, with the fluorescence excitation wavelength set at 360 nm and fluorescence intensity data taken at 440 nm (excitation wavelength). Kinetic readings were taken over 5-7 minutes using an LS 55 fluorescence spectrophotometer with triplicate reading per sample used to determine the SD of the data. A standard deviatiation value was not possible for the T0 sample since a single data point was collected.

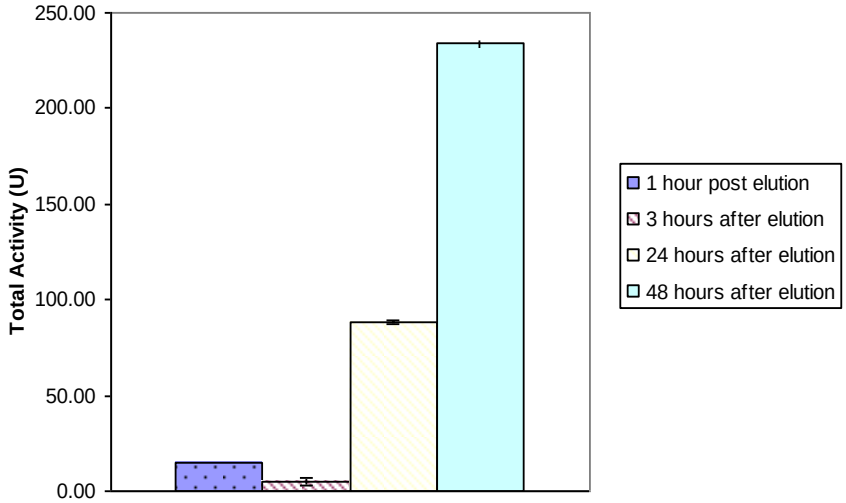


Figure 21. Chitinase elution after substrate affinity purification

Chitinase enzyme was eluted after substrate complexing by incubating the bound enzyme at 30^{o}C for 48 hours with gentle agitation (90 rpm on an orbital shaker). Samples were taken at 1 hour, 3 hours, 24 hours as well as 48 hours and analysed for chitinase activity using 10 µM 4-MU CHB. The fluorescence excitation wavelength was set at 360 nm and emission data was gathered at 440 nm. Kinetic readings were done over a 5-7 minute incubation period with triplicate readings used to determine the SD of the data.

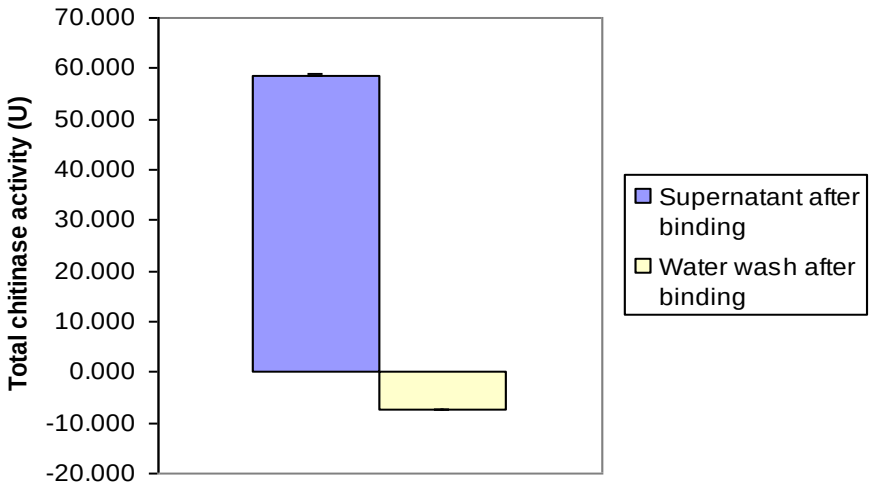


Figure 22. Supernatant and water wash chitinase activities after substrate binding

Chitinase activities were assayed using 10 µM 4-MU CHB as substrate. Kinetic readings were taken using an LS 55 fluorescence spectrophotometer, with triplicate readings averaged and extrapolated to determine the SD of the data. The excitation wavelength was set at 360 nm while the emission wavelength was 440 nm. Slit widths were set at 10 nm with the scan speed being 400 nm/second.

2.5.5 Assessment of different ion exchange chromatography conditions

Enzyme purification results show that anion EX resins performed better in purification studies when compared to cation EX resins (see Appendix 6: Figure 39 to Figure 47 for the individual chromatographic profiles). None of the cation EX resin fractions showed chitinase activity whereas the anion EX resin fractions showed activity when tested against 4-MU CHB with the HiTrap QFF fractions giving the best results (fraction IV-specific activity 10.3 µmol/ mg/mL with a 37% yield on chitinase activity)(Table 2).

To validate the results obtained a second purification run was done testing the HiTrap QFF resin. Results from fraction IV showed a 2.7 fold increase in specific activity when compared to the original post-dialysis sample applied to the column (Table 3). Since the QFF Fastflow column was found to give the best recovery of chitinase activity with the least quantity of contaminating protein, all subsequent purification work involved the use of this resin.

Table 2. Specific activity of cationic as well as anionic exchange chromatography fractions

Descriptive	Activity Units/ mL	Total activity	[Protein] mg/mL	Specific activity Units/mg protein	% Yield
CFE concentrate	14.17	14.17	2.22	6.38	100.00
HiTrap SPP	-		-	-	-
HiTrap CMFF F4	-		0.004	-	-
HiTrap CMFF F5	-		0.12-	-	-
HiTrap SPXL	-		-	-	-
HiTrap DEAE FF	0.44	6.16	0.10	4.29	96.5
HiTrap QFF fraction IV	**0.48**	**2.40**	**0.05**	**10.30**	**37.62**
HiTrap QFF fraction V-VII	0.13	1.95	0.13	1.00	30.56
HiTrap ANX FF	0.16	3.04	0.04	4.27	47.64
HiTrap QXL fraction IV	0.15	0.75	0.09	1.70	11.75
HiTrap QXL fraction V-VII	0.11	1.65	0.05	2.24	25.86

- No protein or activity detected

Approximately 4 mg of the CFE concentrate was applied onto both anionic as well as cationic exchange columns. Chromatographic conditions were as follows: (1) for anion EX chromatography the start buffer used was 20 mM Tris (pH 9.0) while the elution buffer contained 1 M NaCl added to the start buffer composition (HiTrap DEAE FF, HiTrap QFF, HiTrap ANX as well as HiTrap QXL); (2) for cation EX chromatography the start buffer employed was 20 mM MES (pH 6.0) while the elution buffer used contained 1 M NaCl added to the respective start buffer (HiTrap SPP, HiTrap CMFF, HiTrap SPXL). The column flow rate was set at 1 mL/min, with 5 mL fractions collected and combined when necessary and assessed (based on protein detection). Protein quantification was done using the Bradford technique while chitinase activity was assessed against 10 µM 4-MU CHB. Reactions were run at 25^{o}C with intermittent mixing. Fluorescence was measured using the LS-55 Perkin Elmer Fluorescence Spectrophotometer, with sample excitation at 360 nm and sample emission measured at 440 nm. Averages of triplicate readings were used to calculate the respective reaction rates. All reactions contained 10 µM 4-MU CHB as substrate.

Table 3. Chitinase unit activities after chromatographic purification using HiTrap QFF column

Descriptive	Reaction rate (min^{-1})	µmoles 4-MU	Protein concentration (mg/mL)	Dilution factor	Unit/mL	Specific activity
Sample Post Dialysis	148.22	0.05	2.63	500	27.73	10.54
QFF Fraction 4	**61.93**	**0.02**	**0.04**	**50**	**1.01**	**27.12**
QFF Fraction 5-8	75.86	0.03	0.16	50	1.30	7.95
QFF Fraction 9-22	22.47	0.004	0.08	50	0.21	2.73
QFF Waste fraction	24.59	0.005	0.04	50	0.25	6.96

Approximately 4 mg of a cell free protein concentrate was applied onto a 1 mL HiTrap QFF anion EX resin column connected to the AKTA prime protein purification system. Protein fractionation was done using a shallow 1 M NaCl gradient in 100 mM Tris (pH 9.0) set at a flow rate of 1 mL/min with 5 mL fractions collected and analyzed. Chitinase activities were determined using 4-MU CHB as substrate. Reactions proceeded at 25^{o}C with intermittent mixing and readings were evaluated every 60 seconds over a 5 minute reaction period using the LS 55 fluorescence spectrophotometer (excitation wavelength set at 360 nm, emission wavelength at 440 nm). Averages of triplicate readings were used to calculate the respective reaction rates. Protein concentrations were determined using the Bradford protein quantification method (Bradford 1976). All reactions contained 10 µM 4-MU CHB as substrate.

Anion EX chromatography purification of the chitin affinity purified enzyme sample was carried out using a 25 mL QFF fast flow column from GE Healthcare. The chitinase enzyme in the sample was eluted from the column using a linear 1M NaCl elution buffer with chitinase activity against 4-MU CHB observed in fractions 8-12 which collected at ~30-35% conductivity (Figure 24) (see Figure 23 for a bar graph of the total chitinase activity plotted against sample specific activity).

A final assessment of the result of each of the purification steps can be viewed in the purification table below (Table 4).

The proteins eluted after chitin-affinity purification was in the region of 35-45 kDa in size (Figure 25) (see Appendix 7 for sample and gel preparation methods used). The final chitinase activity was purified to a final yield of 7% with a 107 fold purification of the extracellular fraction. The purity of the extracellular chitinase enzyme sample was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The sample did not show the appearance of any smaller protein products/ subunits after enzyme heat treatment (see Figure 25; lane 5 sample).

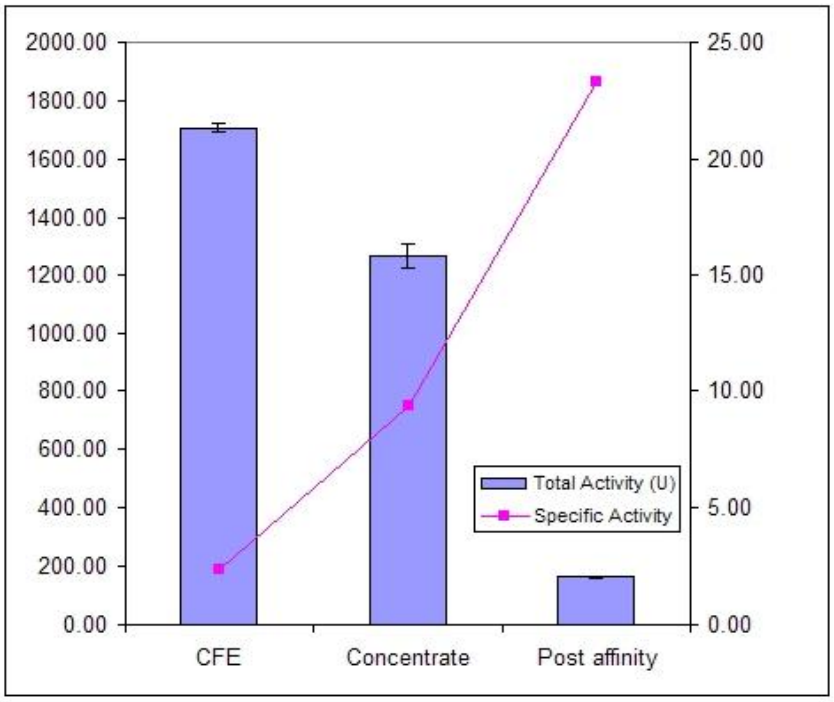


Figure 23. Graph of total chitinase activity (units) versus chitinase specific activity

CFE, concentrate as well as affinity purified chitinase enzyme samples were assayed using 10uM CHB as substrate.

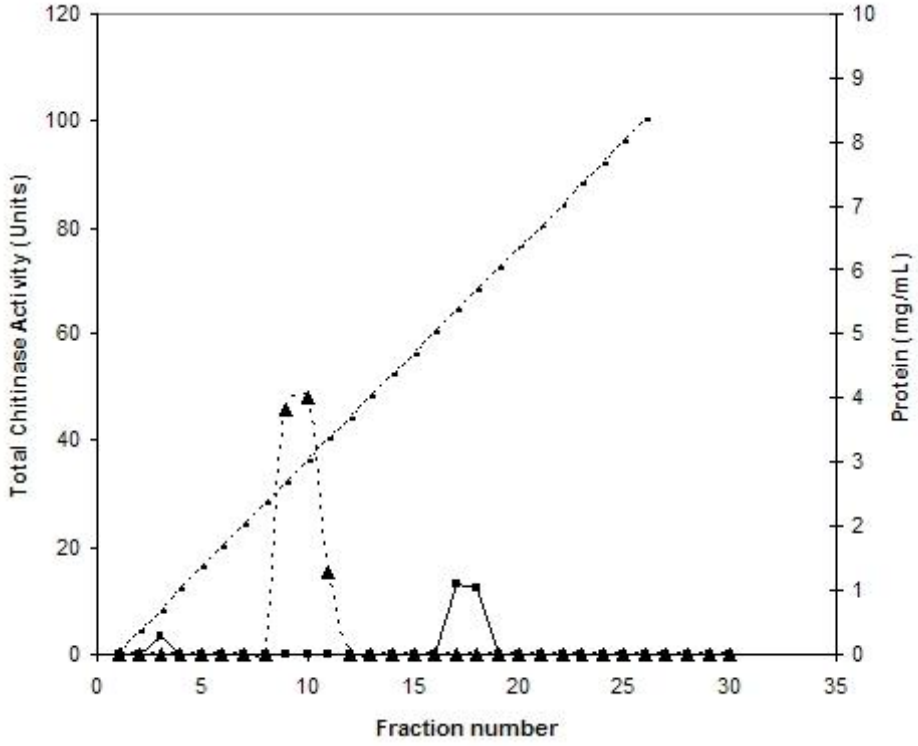


Figure 24. Anion EX chromatography elution profile of the extracellular chitinase from strain PBY

▲ - Chitinase activity using 4-MU CHB as substrate; ■ - Protein concentration in sample using the Bradford method of quantification; ▪ - NaCl gradient (1M from 0-100%). Protein purification was done using a QFF fast flow anion exchange column connected to an AKTA purification system with a linear salt gradient (0- 1 M) introduced into the system to elute column bound proteins. Fractionating of sample was done using a flow rate of 1 mL/ min with individual fraction volumes being 5 mL.

Table 4. Chitinase enzyme purification table

Purification Step	Total Activity (U)	Total Protein (mg)	Specific Activity	Fold purification	Yield
CFE	1707.67	714.6	2.39	1	100
Concentrate Post Diafiltration	1262.79	134.9	9.36	3.92	73.95
Chitin Affinity Purification	163.13	15	10.87	4.55	9.55
Post QFF purification	119.8	0.469	255.44	106.8	7.01

Activity data was obtained using 10 µM CHB. Fluorescence spectrophotometer readings were assessed to determine individual reaction rates, with 4-MU µmol concentrations determined using the prepared 4-MU standard curve. All data showed originated from triplicate samples that were analysed with the calculated average data used to determine the chitinase activity value/s One unit of activity is defined as the amount of enzyme required to release 1 µmol of 4-MU into solution/ minute (25^{o}C, 100 mM phosphate buffer, pH 7.0).

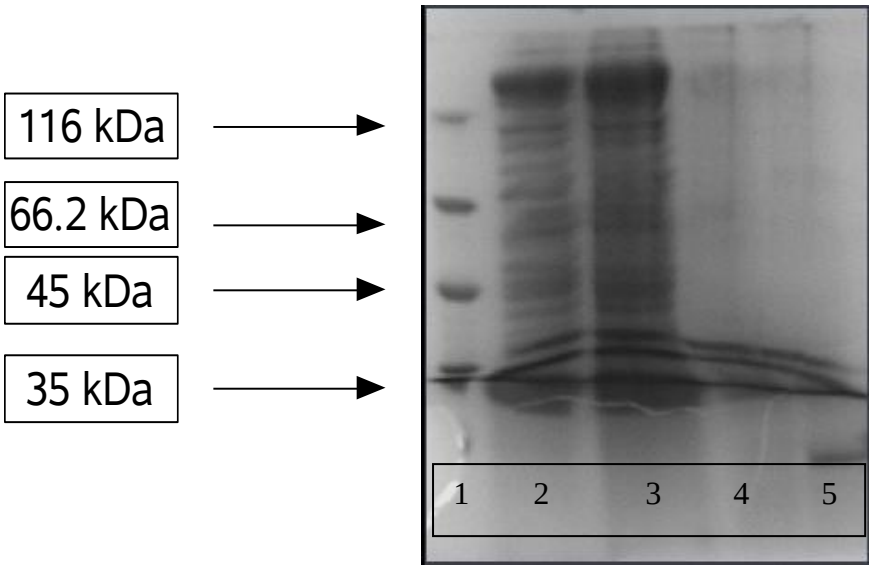


Figure 25. SDS PAGE of CFE post affinity purification

From left, Lane:-1 Fermentas SM 0431 Protein Molecular Weight Marker;

Lane 2:- CFE post centrifugation

Lane 3:–CFE Concentrate post Dialysis

Lane 4:- Sample post chitin-affinity purification showing the eluted chitinolytic enzyme/s (~35-40 kDa in size)

Lane 5:- Heat treated enzyme sample post chitin-affinity purification. Samples were run resolved using a 10% SDS-PAGE with the gel run using a constant voltage (100V).

Finally in terms of chitinase activity purification, a combined strategy of affinity purification followed by anion EX chromatography provided complete purification and separation of the chitinolytic enzymes produced by strain PBY under the induction conditions tested.

2.6 Conclusion

The chitinase producing environmental isolate purified using a selective *Bacillus* enrichment protocol was identified as belonging to the genus *Paenibacillus* with the strain designation by DNA hybridisation studies being a *P. chitinolyticus* (>99% sequence homology). Morphological characterisation of the cells by microscopy show that cells are typically rod shaped with the mean cell size being ~4.26 µm. Cells are visibly motile with motilility documented to be as a result of peritrichous flagella.

Chitinase production was shown to be induced in the presence of pure chitin rather than produced constitutively by strain PBY. The observed increase in chitinase activity in Figure 15 shows a 16 fold increase in chitinase activity in solution from day one to day three. This cannot be the result of biomass production in minimal media since cell bulking up was done on TSB prior to the addition of cells into the minimal media containing chitin. There also aren't any expectations that much more biomass was generated in the minimal defined media which contains small amounts of yeast extract, chitin and calcium carbonate.

Chitinase production was successfully induced within 48-72 hours using the minimal media mentioned before with chitinase induction in someway resulting in a change in cell colour from cream to bright yellow.

The cell doubling time of strain PBY in TSB during logarithmic growth was found to be 50 minutes. No growth curves on the minimal media were done due to interference of the chitin in the media. The only documented growth curve studies for comparative purposes was done on some novel *Streptomyces* strains which showed the average cell doubling time on enrichment media to be 3.7, 11.6, 2.4, 6.3 and 3.7 hours for strains SGE2, SGE4, SSL3, MG1 and MG3 respectively (Hoster *et al.* 2005). Generally cell doubling times of less than an hour are favourable since cell density increases are possible over shorter incubation time periods. It is therefore adequate for cells to have been cultured overnight prior to transfer into the minimal media.

Looking at the purification protocol followed for isolation of the chitinase enzyme, the extracellular fraction after concentration and diafiltration showed a four-fold increase in purification with a reduction in overall chitinase yield of 26% (~1707 U in CFE versus 1263 U in concentrate). The concentrate when subjected to substrate affinity purification using pure chitin resulted in the recovery of ~7% of the initial activity present in the original enzyme concentrate used. This is lower than the documented percentage chitinase recovery after substrate affinity purification obtained from work done on *Vibrio carchariae* (Suginta *et al.* 2000) and may be due to inadequate chitin digestion with much of the chitinase enzyme still possibly bound onto the substrate. An increase in incubation temperature for digestion could improve the chitinase recovery from the substrate. It is also possible that some activity loss could have been incurred during the enzyme binding and elution steps.

CHAPTER THREE

CHITINASE ENZYME CHARACTERISATION FROM AN ISOLATED ENVIRONMENTAL STRAIN OF *P. CHITINOLYTICUS*

3 CHAPTER THREE - CHITINASE ENZYME CHARACTERISATION FROM AN ENVIRONMENTAL STRAIN OF *P. CHITINOLYTICUS*

3.1 Abstract

A novel environmental strain PBY which was previously isolated from an environmental soil sample by chitin media enrichment was found to produce multiple extracellular chitinolytic enzymes under the chitinase induction conditions tested. Characterisation of the dominant chitinolytic enzyme, which has a pI of ~7.0, showed the extracellular chitinase functioned optimally at close to ambient temperature conditions. The temperature stability data obtained showed that the enzyme retained 50% chitinase activity after an hour of incubation at 65ºC. The chitinase enzyme showed optimum activity between pH 6.5-7.5 while substrate hydrolysis studies showed that the enzyme only hydrolysed substrates having a minimum of three chito-saccharide residues in size (including any aglycon residues present). The fluorescent substrate 4-MU CHB which only contains two chito-saccharide residues was found to be hydrolysed by the enzyme, however activity is most likely due to the *O*-glycosyl 4-MU residue in the substrate being recognised as a sacchride residue linkage. The enzyme showed a distinct preference for longer chain substrates with the K_m for 4-MU CHB being 7.45 µM 4-MU versus 5.72 µM 4-MU for 4-MU CHT.

3.2 Introduction

The physiological properties of chitinases produced by microbial sources generally vary in their pH and temperature optima based on the substrates employed during the enzyme characterisation studies. As a general guide pH optimums of chitinases from microbial cultures characterised vary from 4.0-9.0 with *Bacillus* sp. BG-11, *Bacillus* sp. 13.26 and *Bacillus* NCTU2 strains showing best activity at pH 7.0-9.0 (Bhushan and Hoondal 1998); (Yuli *et al.* 2004); (Wen *et al.* 2002). The chitinase temperature optima in the same studied population ranged between 30-60^{o}C, with the *Bacillus* sp enzymes having an optimum temperature of 45-60^{o}C.

In terms of substrate length, the longer chain substrates are generally considered the real substrates for chitinases whereas the shorter substrates tend to be the reaction products. The K_m values for microbial chitinases range between 0.47-2.85 mg/mL which on average is higher than those documented for insect, crustacean as well as plant chitinases. This indicates that microbial chitinases tend to have a lower affinity towards the substrates studied (Koga *et al.* 1999).

Microbial chitinases hydrolyse chitin to produce products of various lengths and patterns. Some split substrates in an endo fashion while others split single or dimeric residues from the reducing end of the substrate. In general definition chitinases split chitin in a random manner.

Finally the pI values of chitinases in microorganisms lie in the range of 3.5-8.8 with some authors documenting the presence of multiple chitinase isoforms in a single organism (Watanabe *et al.* 1990).

3.2.1 Aims

- To determine the kinetic properties (V_{max}, K_m) of the purified chitinase
- To determine the operational pH range as well as optimum pH of the chitinase
- To determine the operational temperature range as well as the enzyme stability at elevated temperatures
- To determine the enzyme thermal half life
- To demonstrate the presence of chitinase isozymes by two-dimensional polyacrylamide electrophoresis (2-D PAGE) and zymography
- To ascertain the nature of the chitinase enzyme produced, i.e. exochitinase or endochitinase
- To compare the kinetic data and operational working parameters obtained from strain PBY to available documented information

3.3 Materials and Methods

3.3.1 Iso-electric focusing (IEF) of a partially purified enzyme suspension from strain PBY

Approximately 0.1 mg of the crude protein was dissolved in 500 µL of a multiple-chaotrophic rehydration buffer comprising 6 M urea, 4% (w/v) CHAPS, 0.5% (v/v) ampholyte solution, 20 mM dithiothreitol (DTT) and 0.002% (w/v) bromophenol blue. The incorporation of urea and glycerol in the rehydration buffer was to diminish the effect of electro endosmosis and to improve protein transfer from the strip to the second dimension. DTT was added to preserve the reduced state of the denatured unalkylated proteins while bromophenol blue allowed for the protein migration through the gel to be observed.

The sample was introduced by diffusion onto a linear 13 cm, pH 3 to 10 immobilised pH gradient (IPG) strip. After sample addition the strip was covered in a thin layer of mineral oil to prevent evaporation of the sample during focusing. The strip was rehydrated and focused using the following protocol: 50 V-12 hrs; 500 V-1 hr; 1000 V-1 hr and finally 8000 V-6 hrs. The strip once focused was removed and equilibrated using an sodium dodecyl sulphate (SDS) equilibration buffer consisting of 50 mM Tris (pH 8.8), 5 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue made up to 200 mL using deionised water. DTT was added into the SDS equilibration buffer only before used. The equilibration buffer was used to maintain the IPG strip pH in the required range for electrophoretic separation.

3.3.2 Second dimensional separation of the focused protein sample (2D PAGE)

Second dimensional separation of the focused protein sample was done by electrophoresis using a 10% polyacrylamide gel. The resolving gel after electrophoresis and post equilibration by gel immersion in 100 mM Tris, pH 7.0 (10 to 15 minute incubation to allow for pH adjustment prior to zymography) was incubated with a substrate overlay gel containing 0.1% (v/v) glycol chitin and 0.8% (w/v) agarose made up to volume in 100 mM Tris buffer, pH 7.0 (Trudel and Asselin 1989). Glycol chitin

was prepared from glycol chitosan purchased from Sigma-Aldrich (Molano *et al.* 1977). Both the resolving protein and substrate gels were left to incubate at 37^{o}C for 2-3 hours and then separated, with the overlay gel being flooded with a Congo-red solution (1 mg/mL solution) and the resolving protein gel stained with Coomasie Brilliant Blue R-250. Staining was done on a shaker for 5 to 10 minutes to enable even staining. De-staining of the overlay gel was done using a 0.5 M NaCl solution while the resolving protein gel was destained using an acetic acid/methanol destaining solution. Diffusion zones indicating chitinolytic activity were visualised as zones of clearing against a red background.

The interaction of Congo red with intact β-D-glucans was used as a colorimetric method for detection of chitinase activity (Teather and Wood 1982). Visualisation of chitinolytic activity in the substrate overlay gel was then possible (Hirano *et al.* 1976; Molano *et al.* 1977).

3.3.3 Testing varied substrate concentrations

A range of varied 4-MU CHB concentrations was used to determine the K_m and V_{max} of the chitinase enzyme. Substrate concentrations ranged from 1 to 40 µM; the protein concentration used was standardized at 4 µg/ mL reaction volume with all samples being prepared in 100 mM phosphate buffer, pH 7.0. Kinetic readings were taken at 60 second intervals for 5 to 10 minutes depending on the individual reaction rates.

3.3.4 Kinetic plots evaluated

A number of varied kinetic plots were used to calculate both the V_{max} as well as K_m of the enzyme tested. This included the use of (1) the Michaelis-Menten graph, (2) the Lineweaver-Burk graph; (3) the Hanes-Woolf graph, and (4) the Eadie-Hofstee graph. The V_{max} and K_m kinetic parameters were calculated under conditions of changing substrate concentrations (1-40 µM substrate concentration).

3.3.5 Statistical analysis of data

The method of least squares was used to determine the vertical deviation for graphical data obtained. Reproducibility of data was done using the students T-test, which is

often used to express confidence intervals when comparing data from differing experiments (Harris 1995). The standard error of the mean (SEM) was calculated as follows: SEM = SD/√n (where SD is the calculated standard deviation of the data points and *n* is the number of data points in the study). The data variance (VAR) was calculated as follows: VAR =SD^2. By definition data VAR provides information on the spread of the data.

3.3.6 Determining the effect of pH on relative chitinase activity

The effect of pH on relative chitinase activity was ascertained by using a universal buffer comprising 100 mM sodium di-hydrogen orthophosphate, 100 mM Tris and 100 mM acetic acid. The pH values tested ranged from 4.0 to 9.0 with 0.5 unit increments between pH values. Kinetic activities were done using 10 µM 4-MU CHB. Each reaction contained 10 µM substrate and 29 µg enzyme /mL reaction volume in a 100 mM phosphate buffer at pH 7.0.

Fluorescence readings were commenced without enzyme to obtain blank values with enzyme addition thereafter. The amount of 4-MU released was measured using an LS-55 Perkin Elmer Fluorescence Spectrophotometer with the excitation wavelength set at 360 nm and emission wavelength readings taken at 440 nm. Slit widths of 10 nm were used and the scan speed was set at 400 nm / second. Reactions were run for 8 to 10 minutes (depending on the reaction rate) with the reaction rate data obtained used to extrapolate the optimum pH for chitinase activity.

3.3.7 Determining the stability of the chitinase enzyme at elevated temperatures

Enzyme stability profiling was done to determine the effect of temperature variance on chitinase activity. Evaluating the temperature profile would indicate the effect of temperature on protein structure and function, and thus allow some indication of the possible uses and limitations of the said enzyme.

The effect of temperature on chitinase activity was ascertained using a range of temperatures from 10^{o}C to 90^{o}C, increasing in 10^{o}C increments. Enzyme suspensions

containing 200 µg of the enzyme per mL phosphate buffer were incubated at varied temperatures for an hour prior to assaying.

The temperature treated enzyme solutions were removed after incubation and immediately tested for activity by adding 29 µg enzyme / mL reaction volume to pre-prepared buffer substrate mixes comprising 10 µM 4–MU CHB and 100 mM phosphate buffer, pH 7.0. Duplicate samples were evaluated with readings taken per minute over a 5 minute incubation period with intermittent mixing of the reaction contents at 25^{o}C temperature. The data obtained was used to calculate chitinase reaction rates as well as unit. Fluorescence readings were commenced without enzyme to obtain blank values with enzyme addition thereafter. The amount of 4-MU released was measured with the excitation wavelength set at 360 nm and emission wavelength readings taken at 440 nm. Slit widths of 10 nm were used and the scan speed was set at 400 nm/ second.

3.3.8 Determining the optimum temperature for relative chitinase activity

Enzyme temperature profiling was done to determine the temperature parameters under which the enzyme could function optimally. The temperature profile would serve to indicate the possible effect of temperature on the extracellular chitinase enzyme should the enzyme be considered promising for a possible biochemical synthesis process or for agricultural application studies.

A 200 µg/mL enzyme suspension was prepared and used for the individual temperature trials. Reactions were commenced by the addition of 29 µg/mL of enzyme into pre-prepared buffer and substrate mixes which were incubated for 5 minutes at temperatures ranging from 10^{o}C to 90^{o}C, with agitation on an Eppendorf shaker (reaction mixtures contained 10 µM 4-MU CHB in 100 mM phosphate, pH 7.0). Kinetic readings were then taken at 60 second intervals at ambient temperature, with data obtained used to plot a curve of activity.

3.3.9 Profiling substrate hydrolysis using thin layer chromatography (TLC)

Profiling of the chitinase from strain PBY was done by studying the hydrolysis products formed after incubation of the enzyme suspension with the following substrates:

GlcNAc, CHB, CHT as well as CHP. Each reaction mixture contained 1.36 mM of the above-mentioned substrates individually solubilised in methanol/DMF prior to being made up to volume in 100 mM phosphate buffer, pH 7.0. The reactions were commenced by the addition of 20 µg of the enzyme suspension with samples being incubated at 37^{o}C on an agimatic shaker set at 1000 rpm. Reaction samples (3 µL volumes) were removed at 0 min, 30 min, 60 min, 2 hrs, 4 hrs as well as 24 hrs and immediately stored at 4^{o}C until needed.

TLC analysis of the products was done using a modification of the method by Xia *et al* (Xia *et al.* 2001). The solvent used for migration of the substrates/ reaction products onto the Silica gel TLC plates was an n-butanol: acetic acid: water solvent (3:1:1). Approximately 1 µL of the individual reaction products as well as controls were spotted onto the TLC plates with residual moisture being removed by air drying. The dried plates were then run using the chosen solvent system. After migration of the samples on the TLC plate was complete, the solvent front was marked and the plate air-dried. The plate was then stained using an aniline-diphenylamine reagent (4 mL aniline, 4 g of diphenylamine, 200 mL acetone and 30 mL of 85% phosphoric acid) and the plate baked at 180^{o}C for 3 minutes to visualise the hydrolysis products (Tanaka *et al.* 1999).

3.4 Results and discussion

An initial crude enzyme purification exercise was tested using an anion EX resin and showed the presence of two chitinolytic enzymes with distinct pI's. The anion resin–enzyme complex after incubation was tested for chitinase activity against 4-MU CHB (Figure 26) (1 mg of the crude enzyme dissolved in the required universal buffer pH). All enzyme characterisation work to follow was done on the enzyme showing a pI of ~ 7.0.

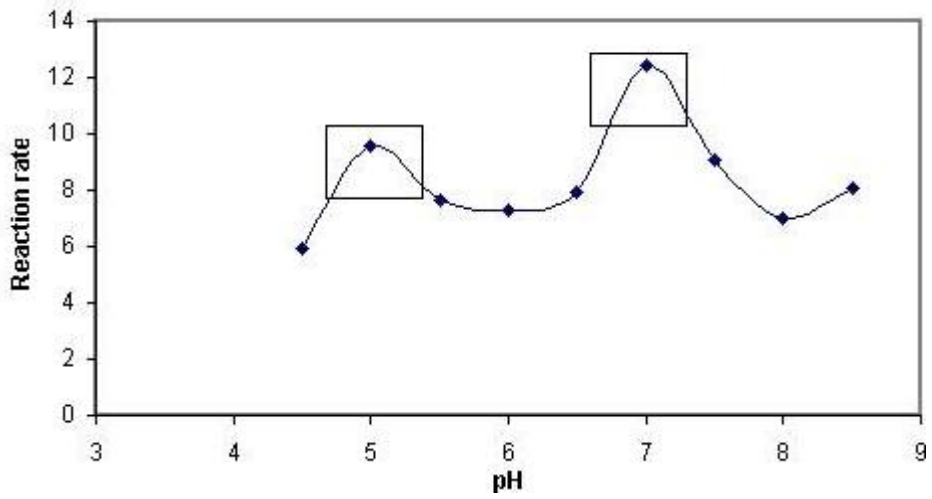


Figure 26. Reaction data obtained from IEX studies done on a crude enzyme preparation from strain PBY

One mg of the crude CFE was dissolved in the universal buffer (pH range from 4.5 to 8.5) and after mixing with 1 g of the anion EX resin was left to stand for 10-15 minutes. The unbound protein fraction was then assayed for chitinase activity using 10 µM CHB as substrate. Readings were taken using a Perkin Elmer fluorescence spectrophotometer with the excitation wavelength set at 360 nm and the emission wavelength at 440 nm. Slit widths were set at 10 nm with the scan speed at 400 nm/ second. Averages of triplicate readings are shown above.

3.4.1 2 – D Protein extract characterisation

2-Dimensional separation of the crude protein extract prepared from strain PBY showed the presence of multiple chitinase isoforms with pI values ranging from 4.8 to 5.3, 5.6, and from 7.0 to 9.2 (see Figure 27 and Figure 28). Previous work done on *Bacillus circulans* WL-12 showed the same multiple nature of chitinases with six major chitinases being detected in the culture supernatant analysed by IEF. The authors showed that the multiple active enzymes were not six distinct genes coding for varied chitinases, but were rather derived from other chitinases which had undergone proteolytic cleavage of the C–terminal portion of the chitinase (N-terminal sequencing of the enzymes showed perfect alignment between chitinase A1 and chitinase A2) (Watanabe *et al.* 1990).

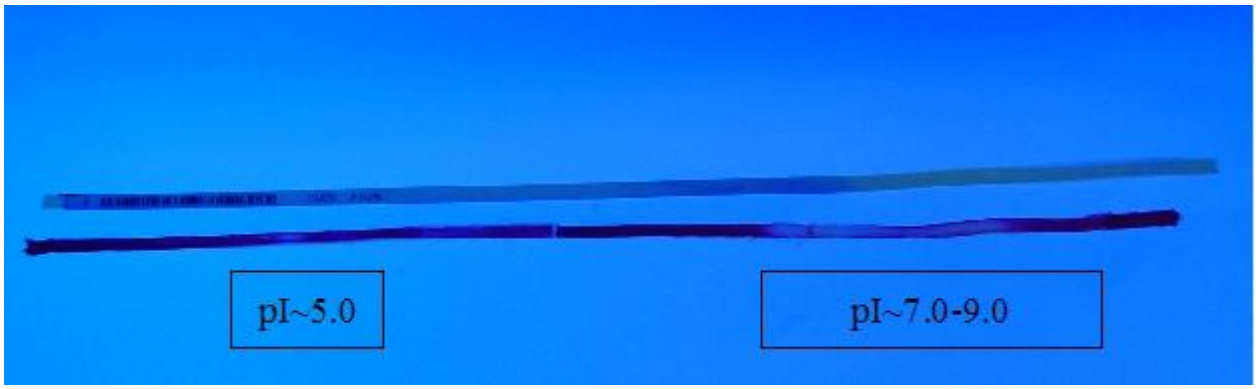


Figure 27. Immobilized pH gradient strip and glycol chitin overlay after Congo red flooding

Protein focusing was done using 0.5 mg of the crude protein preparation after enzyme induction. The CFE was concentrated 10 fold volume reduction by ultrafiltration using a 10 kDa cut-off membrane purchased from PALL. Protein focusing was done using the Amersham IPGphor focusing system. Rehydration of the strip was done at 50 V for 12 hours followed by a stepwise voltage increase as shown: 500 V for 1 hour; 1000 V for 1 hour and finally 8000 V for 6 hours. The protein sample was then solubilised in a multiple chaotrophic buffer comprising 6 M Urea, 20 mM DTT (freshly added prior to use), 0.5% (v/v) ampholyte solution and 4% (w/v) CHAPS. Once focusing was completed the strip was equilibrated using an SDS equilibration buffer comprising 50 mM Tris (pH 8.8), 5 M urea, 30% (v/v) glycerol and 0.002% (w/v) bromophenol blue prior to the addition of the molten glycol chitin solution overlay to the strip. The resulting immobilised protein-substrate sandwich was incubated at 37^{o}C for 3 hours prior to staining of the glycol chitin zymogram using Congo red. Enzyme activity was visualised as a region of clearing against a red-stained background.

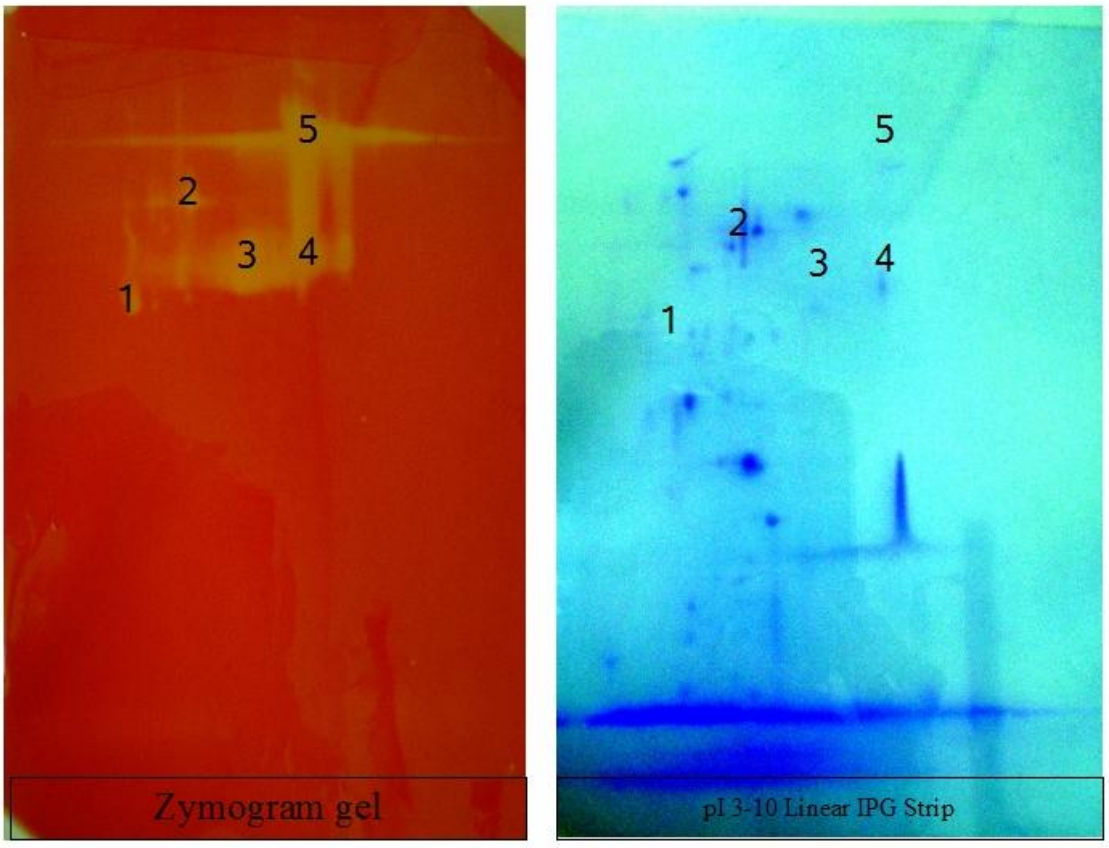


Figure 28. 2-D Protein gel and zymogram showing chitinolytic activity of the separated proteins

Right gel: The focused protein after equilibration was subjected to electrophoresis using a 10% polyacrylamide resolving gel that was run under constant amperage. The gel run time was found to be between 3-4 hours under the tested conditions. Once complete the gel was removed and equilibrated to the correct assaying pH using a 200 mM Tris-Cl buffer (pH 7.0). After incubation with the substrate gel the resolving gel was stained using Coomasie brilliant blue R250 followed by destaining. Left gel: The zymogram gel comprising 0.1% (w/v) glycol chitin and agarose, made up to volume using a 200 mM Tris-Cl buffer (pH 7.0) was flooded with a 0.1% (w/v) Congo-red solution to enable visualisation of the chitinolytic zones. The areas marked off as spots 3, 4 and 5 correspond to a pI ~7.0, whereas the spot marked as 2 corresponds to an pI~5.0.

3.4.2 Kinetic plots using varied 4-MU CHB concentrations

Kinetic data was collected using two varied length substrates, namely 4–MU CHB and 4–MU CHT tabulated data in Appendix 9). The kinetic parameters for chitinase activity was calculated as an average from data extrapolated using the Lineweaver–Burk plot, the Eadie–Hofstee plot as well as the Hanes–Woolf plot. The average K_m values

obtained were 5.72 µM and 7.45 µM for 4-MU CHB and 4–MU CHT respectively while the average V_{max} values were found to be 0.079 µmol 4–MU/ min and 0.135 µmol 4–MU/ min for 4-MU CHB and 4-MU CHT respectively. The K_m value obtained from strain PBY chitinase using 4–MU CHB is similar to that documented from *Clostridium paraputrificum* ChiB as well as from *Streptomyces plicatus* Chi62 (Table 9). The plot data illustrated in Figure 29 and Figure 30 show four varied data interpretation plots however the average V_{max} and K_m values from only three of the four plots are tabulated (see Table 5 and Table 7) Even though the Michaelis-Menten equation can be used to calculate V_{max} and K_m values, these values are often subject to error due to the difficulty of measuring initial reaction rates at high substrate concentrations. The results in Table 5 and Table 7 therefore only provide kinetic data extrapolated from the Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots which are linearization of the Michaelis-Menten equation which allow for normalisation of experimental data (Bravo *et al.* 2001). The Lineweaver-Burk plot shows no deviation from the curve at higher substrate concentrations indicating no substrate concentration inhibition on the enzyme. An upward curve observed at high substrate concentrations in the Lineweaver-Burk plot would indicate substrate concentration inhibition of the enzyme.

A comparative assessment of the varied kinetic plots can be seen in Figure 29 and Figure 30, with data summarised in Table 5 and Table 7 showing similarity between the K_m and V_{max} values obtained using the different plot methods (Cornish-Bowden 1995) (see Appendix 8, Table 16 to Table 19 for the raw data used to construct the plots shown in Figure 29 and Figure 30). Statistical analysis of the data obtained using the varied data normalisation methods can be viewed in Table 6 and Table 8. The Eadie-Hofstee plot data observed when incubating the extracellular chitinase with 4-MU CHB gave a significant variance from the best fit curve. A common concern with the Eadie-Hofstee plot is that the x and y axis values, which should be independent values, often carry an element of the velocity of the reaction therefore any experimental error that occurs will be observed on both data axes.

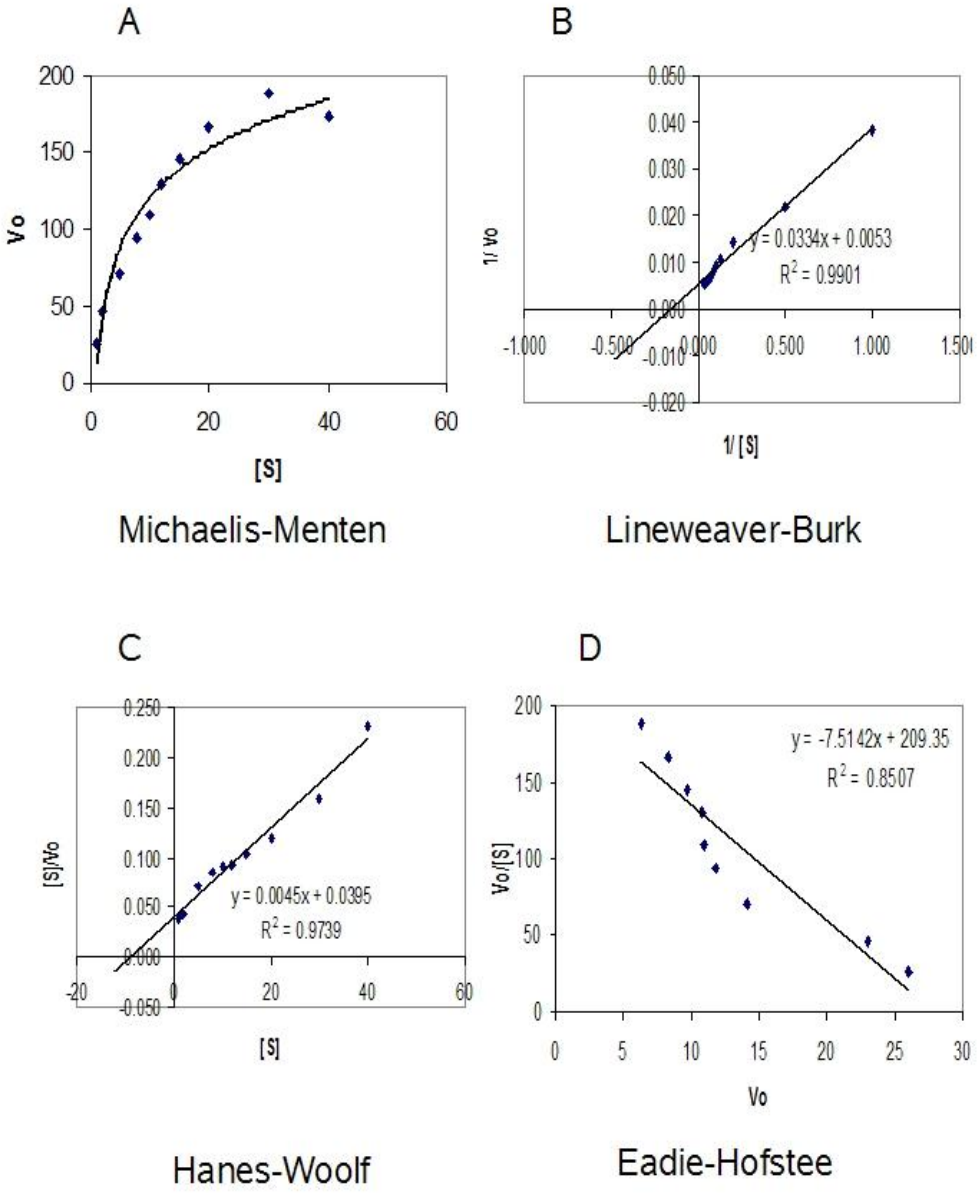


Figure 29. Graphs of kinetic plot data obtained using 4-MU CHB as substrate

Kinetic plots obtained using varied data plotting methods with 1 µM to 20 µM 4-MU CHB being used as substrate (A)–Michaelis-Menten Plot, (B)-Lineweaver-Burk Plot, (C)–Hanes-Woolf Plot, (D)–Eadie-Hofstee Plot. [S] Represents substrate concentration while Vo represents reaction velocity. Varied kinetic plots obtained using 4-MU CHB as substrate showed good least square regression values from triplicate data point readings.

Table 5. Comparative V_{max} and K_m values using 4-MU CHB as substrate

Comparative V_{max} and K_m values obtained using varied plot methods

Method employed	V_{max} (µmoles 4-MU/min)	K_m (µM 4-MU CHB)
Eadie-Hofstee plot	0.080	7.51
Hanes plot	0.085	8.77
Lineweaver Burk plot	0.072	6.31
∑	**0.079**	**7.53**

Statistical analysis of the K_m and V_{max} values obtained via the three varied data extrapolation plots suggests the following: (1) with a 95% confidence level the K_m value can be said to reside between 4.47 µM and 10.58 µM; with the variance of the data population being 1.513; (2) with a 95% confidence level the V_{max} can be said to be found between 63 and 95 nM 4-MU/ min (Table 6).

Table 6. Statistical analysis of kinetic data obtained from 4-MU CHB

Data	∑	SD	n	SEM	Data variance	t test value (95% confidence interval)	Range
K_m	7.53 µM	1.23	3	0.710	1.513	4.303	4.47-10.58
V_{max}	0.079 µmol/min	0.006	3	0.004	4.3×10^{-5}	4.303	0.06-0.09

Statistical evaluation of kinetic data obtained using varied kinetic plots. Values calculated below indicate percentage confidence of the studied value/s occupying a place within a specific numeric range. ∑ is the arithmetic mean, SD is the calculated standard deviation, ***n*** is the number of data points available, SEM is the standard error of the mean, Variance is the data variance, and the **t** value taken off the students T-table (Harris 1995).

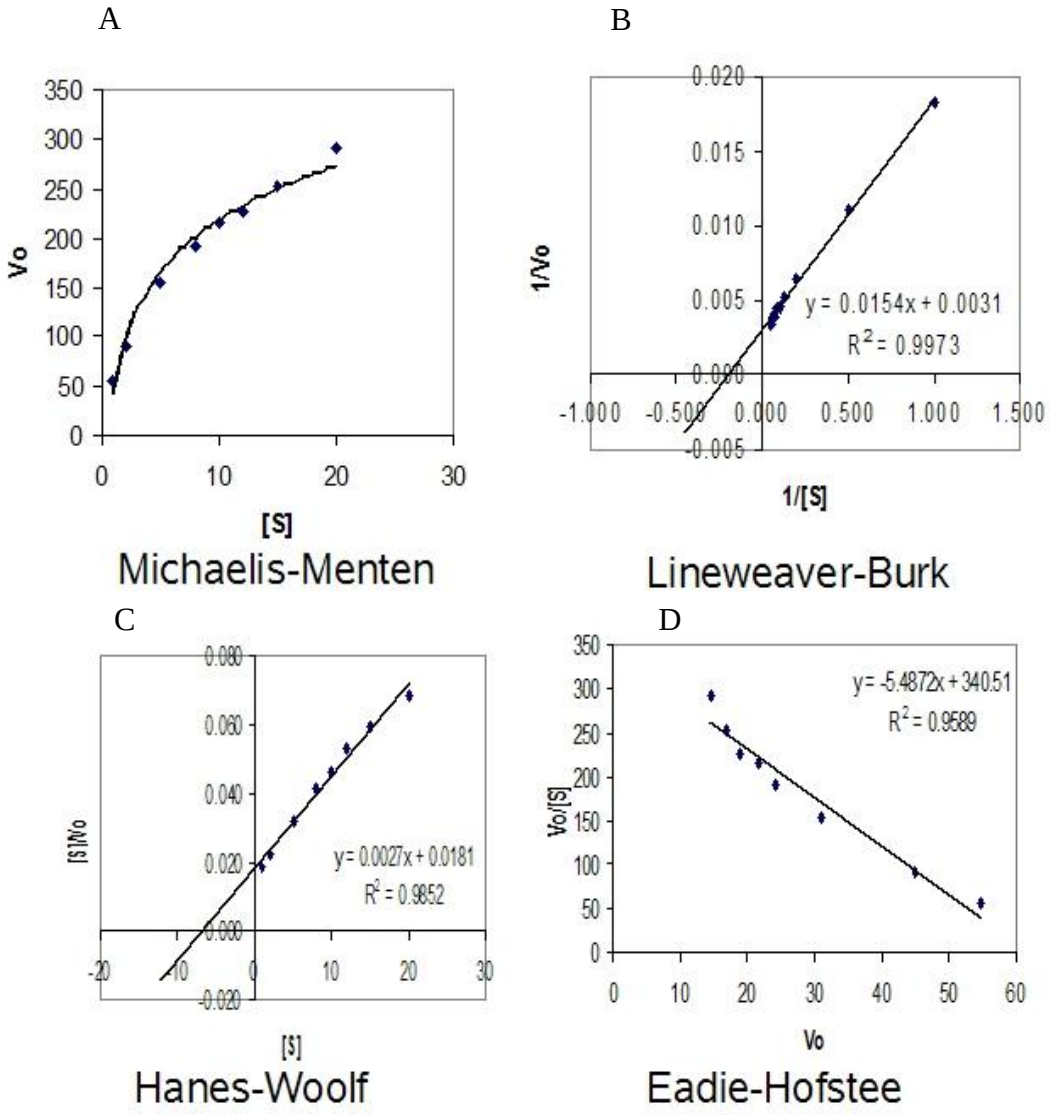


Figure 30. Graphs of kinetic plot data obtained using data from 4-MU CHT substrate reactions

Kinetic plots obtained using varied data plotting methods with 1 µM to 20 µM 4-MU CHT being used as substrate (A)–Michaelis-Menten Plot, (B)-Lineweaver-Burk Plot, (C)–Hanes-Woolf Plot and (D)–Eadie-Hofstee Plot. [S] Represents substrate concentration while Vo represents reaction velocity. Varied kinetic plots obtained using chitotrioside as substrate showed good least square regression values from triplicate data point readings.

Table 7. Comparative V_{max} and K_m values using 4-MU CHT as substrate

Kinetic plot employed	V_{max} (µmoles 4-MU / minute)	K_m (µM 4-MU CHT)
Lineweaver Burk plot	0.126	4.98
Hanes–Woolf plot	0.146	6.70
Eadie–Hofstee plot	0.133	5.48
Mean	**0.135**	**5.72**

Comparative V_{max} and K_m values using varied plots

Statistical analysis of the K_m and V_{max} values obtained via the three studied kinetic plots using 4-MU CHT as substrate suggests the following: (1) with a 95% confidence level the K_m value can be said to reside between 3.52 µM and 7.92 µM; with the variance of the data population being 0.78; (2) with a 95% confidence the V_{max} can be said to be found between 110 and 160 nM 4-MU/ min with the variance of the SD data being $1.103x\ 10^{-3}$ (Table 8)

Table 8. Statistical analysis of kinetic data obtained from 4-MU CHT

Data	∑	SD	n	SEM	Data variance	t test value (95% confidence interval)	Range
K_m	5.72 µM	0.884	3	0.510	0.783	4.303 (95%)	3.52-7.92
V_{max}	0.135µmol/min	0.010	3	0.004	$1.103x10^{-3}$	4.303 (95%)	0.11-0.16

Statistical evaluation of kinetic data obtained using varied kinetic plots. Values calculated below indicate percentage confidence of the studied value/s occupying a place within a specific numeric range. ∑ Is the arithmetic mean, SD is the calculated standard deviation, ***n*** is the number of data points available, SEM is the standard error of the mean, Variance is the SD data, and the **t** value taken off the students T-table (Harris 1995).

3.4.3 Chitinase pH profile

The effect of pH on chitinase activity was assessed by using a universal buffer solution with assaying done using 4–MU CHB as substrate. The activity maximum against 4–MU CHB was found to be between pH 7.0 and 7.5 (Figure 31). In addition 80 to 100% of the activity was retained between pH 6.5 and 8.0, with a ~20% loss in activity observed between pH 8.0 and 8.5. A 35% loss in activity was observed between pH 8.5 and 9.0 (see Appendix 9, Table 20 for raw data used). This dramatic loss of activity at low pH values could indicate a change in the ionization state of critical amino acid residues.

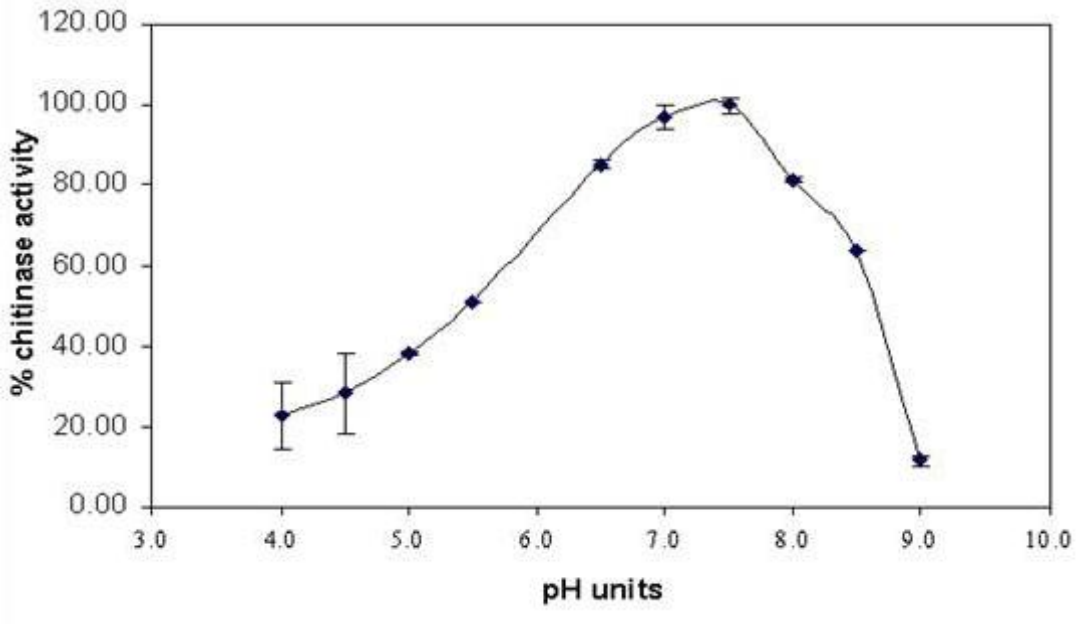


Figure 31. Strain PBY chitinase pH profile

Chitinase pH profile graph showing varied reaction rates when employing different pH buffer environments. Reaction mixes contained 29 µg chitinase together with 10 µM 4-MU CHB which was re-suspended in the respective pH adjusted universal buffer solutions (final buffer concentration was 50 mM). The SD data was calculated from triplicate readings.

3.4.4 Chitinase enzyme stability

Enzyme stability studies showed that the enzyme was stable for an hour between 10^{o}C to 50^{o}C with a significant drop in activity observed at temperatures above 60^{o}C (Figure

32). The enzyme half life at 65°C was calculated to be 1 hour, with no observed chitinolytic activity at temperatures above 80°C (see Appendix 11, Table 22 for raw data used). The observed chitinase enzyme therefore appears function best at ambient to moderately high temperatures.

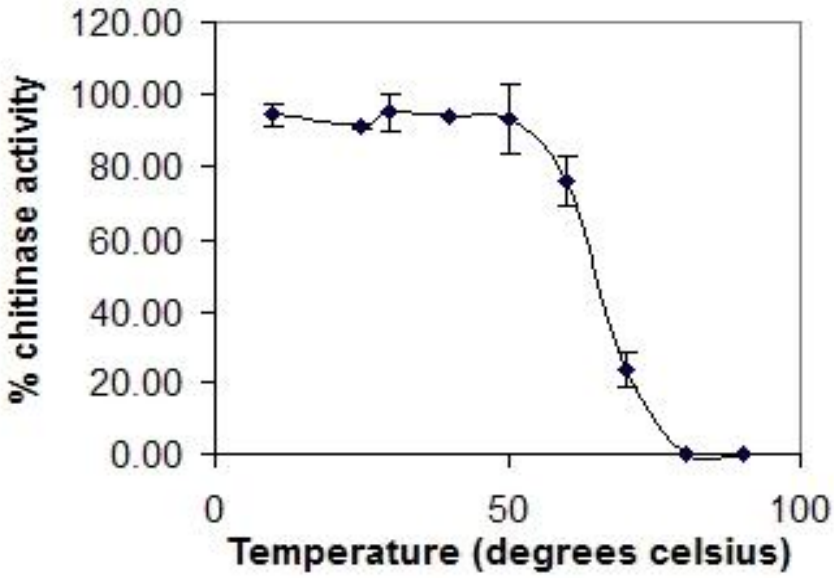


Figure 32. Strain PBY chitinase temperature stability data

Enzyme stability graph using reaction rate data obtained after incubation of the enzyme suspension (1 mg/mL) at the respective study temperatures for a period of 60 minutes. Enzyme-substrate mixtures were incubated at the stipulated temperatures. All reaction mixes contained 29 µg chitinase lyophilisate and 10 µM 4-MU CHB in 100 mM phosphate buffer, pH 7.0. Reaction mixes were incubated on a temperature controlled eppendorf shaker for 5 minutes prior to analysis. Kinetic readings were taken at 60 second intervals over a 5 minute period.

3.4.5 Chitinase temperature profile

Temperature effects on chitinase activity were studied to determine both the optimum temperature of the extracellular chitinase enzyme when incubated at varied temperatures. Results obtained from the temperature profile study done showed maximum chitinase activity at 30°C to 40 °C, with activity maintained at incubation temperatures between 10°C and 40°C (Figure 33). A significant reduction in activity was observed at temperatures above 50°C, however complete enzyme activity loss was

not observed since the enzyme was incubated for five minutes at the evaluated temperatures prior to endpoint assaying. All endpoint data was obtained at ambient temperature since the fluorescence spectrophotometer did not have a peltier system that would have enabled temperature control (Figure 33) (see Appendix 10, Table 21 for raw data).

Total loss of chitinase activity was observed when enzyme suspensions were incubated for an hour at the respective evaluation temperatures (see Figure 32).

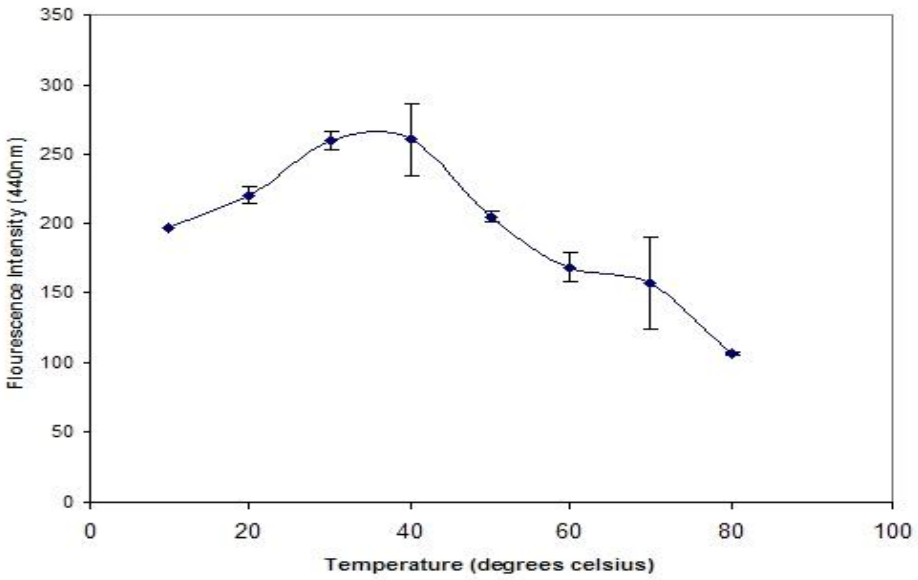


Figure 33. Strain PBY chitinase temperature profile studies

Percentage chitinase activity of reaction mixes incubated at the stipulated temperature/s. Reactions were carried out using 4-MU CHB as substrate with triplicate readings averaged to ascertain the SD of the data. Kinetic assaying was done using the LS 55 fluorescence spectrophotometer with the excitation wavelength at 360 nm and emission data gathered at 440 nm. The scan speed was set at 400 nm/second with slit widths of 10 nm being used.

A comparison of the extracellular chitinase from strain PBY with other documented chitinase enzymes can be seen in Table 9 as well as Table 10. Most chitinase enzymes appear to be produced extracellularly (see Table 10) with many producing endo-acting chitinase enzymes which appear to facilitate hydrolysis of long chain substrates to smaller more soluble chitosaccharides.

Table 9. Kinetic properties of strain PBY chitinase

Microbial chitinases	Substrate	K_m	V_{max}	K_{cat} (per second)	Comment
Strain PBY	**4-MU $GlcNAc_1$**	**-**	**-**	**-**	**No activity**
Strain PBY	**4-MU $GlcNAc_2$**	**7.53 µM**	**1.344 µmol /min/mg**	**224**	**Endo splitting**
Strain PBY	**4-MU $GlcNAc_3$**	**5.71 µM**	**2.288 µmol /min/mg**	**381**	**Endo splitting**
Serratia marcescans BJL2000	4-MU $GlcNAc_2$	34.1 µM	-	-	Exo splitting
Bacillus licheniformis X-7u I	pNp-$GlcNAc_2$	110 µM	3.8 µmol/min/mg	-	Endo splitting
Bacillus licheniformis X-7u II	pNp-$GlcNAc_2$ (pH 6.0)	77 µM	3.1 µmol/min/mg	-	Endo splitting
Bacillus licheniformis X-7u III	pNp-$GlcNAc_2$ (pH 6.0)	50 µM	3.8 µmol/min/mg	-	Endo splitting
Bacillus licheniformis X-7u IV	pNp-$GlcNAc_2$ (pH 6.0)	33 µM	2.9 µmol/min/mg	-	Endo splitting
Clostridium paraputrificum ChiB	4-MU $GlcNAc_2$ (PH 7.0)	6.3 µM	46 µmol/min/mg	-	-
Streptomyces plicatus Chi63	4-MU $GlcNAc_2$ (PH 5.5)	5 µM	-	-	Endo splitting
Streptomyces plicatus Chi63	4-MU $GlcNAc_3$ (PH 5.5)	0.5 µM	-	-	-
Vibrio alginolyticus C1	Squid chitin (pH 7.0)	1.14 mg/mL	-	-	Endo splitting
Vibrio alginolyticus C3	Squid chitin (pH 7.0)	0.8 mg/mL	-	-	Endo splitting

Table 10. Comparative assessment of pH and temperature stability profiles from various prokaryotes

Organism	pH optimum	Localization	Optimum Temperature	Temperature stability	Reference
Strain PBY	**7.0 – 7.5**	**Extra cellular**	**20–40°C**	**65°C (50% activity loss in 1 hour)**	-
Vibrio sp.	10.5 (glycol chitin)	Extra cellular	40°C	60°C (unstable > 60°C)	(Ohtakara *et al.* 1979)
Vibrio sp.	6.0 (colloidal chitin)	Extra cellular	40°C	60°C (unstable > 60°C)	(Ohtakara *et al.* 1979)
Vibrio alginolyticus	9.0 (1st optimum) 4.0 (2nd optimum)	Extra cellular	45°C	40°C	(Murao *et al.* 1992)
Bacillus circulans	8.0	Extra cellular	40°C	-	(Wiwat *et al.* 1999)
Bacillus circulans	3.0	Extra cellular	40°C	-	(Wiwat *et al.* 1999)

3.4.6 Substrate profile of the purified chitinase

In general, chitinases fall into one of two categories: either endo–acting chitinases or exo–acting chitinases. Exochitinases can be divided into two further subcategories: chitobiosidases, which catalyze the progressive release of $GlcNAc_2$ starting at the nonreducing end of the substrate or hexosaminidases, which cleave the oligomeric products of the endochitinase enzymes, releasing monomeric GlcNAc products from the non-reducing end of the sugar. Analysis of the data obtained with strain PBY suggests that the enzyme is an endochitinase for the following reasons: both GlcNAc, $GlcNAc_2$ as well as 4–MU glucosaminide were not hydrolysed even after lengthy incubation periods (16 hours for GlcNAc and CHB and 20 minutes for 4–MU glucosaminide respectively); CHT, CHP, 4–MU CHB as well as 4–MU CHT were hydrolysed by the enzyme with the kinetic data showing a distinct increase in affinity for 4–MU CHT versus 4–MU CHB (K_m for 4–MU CHT–5.72 µM versus K_m for 4–MU CHB–7.45 µM); and finally both CHT as well as CHP incubation with the enzyme resulting in the formation of GlcNAc and CHB concomitantly (see Figure 34 as well as Figure 35). In keeping with the suggestion of the presence of an endo–acting enzyme the positive results obtained with 4–MU CHB can be explained due to the enzyme recognising the structure CHB plus aglycon (methylumbelliferyl moiety) as a trimeric substrate (the aglycon moiety need not be a GlcNAc residue) (Sakai *et al*. 1998).

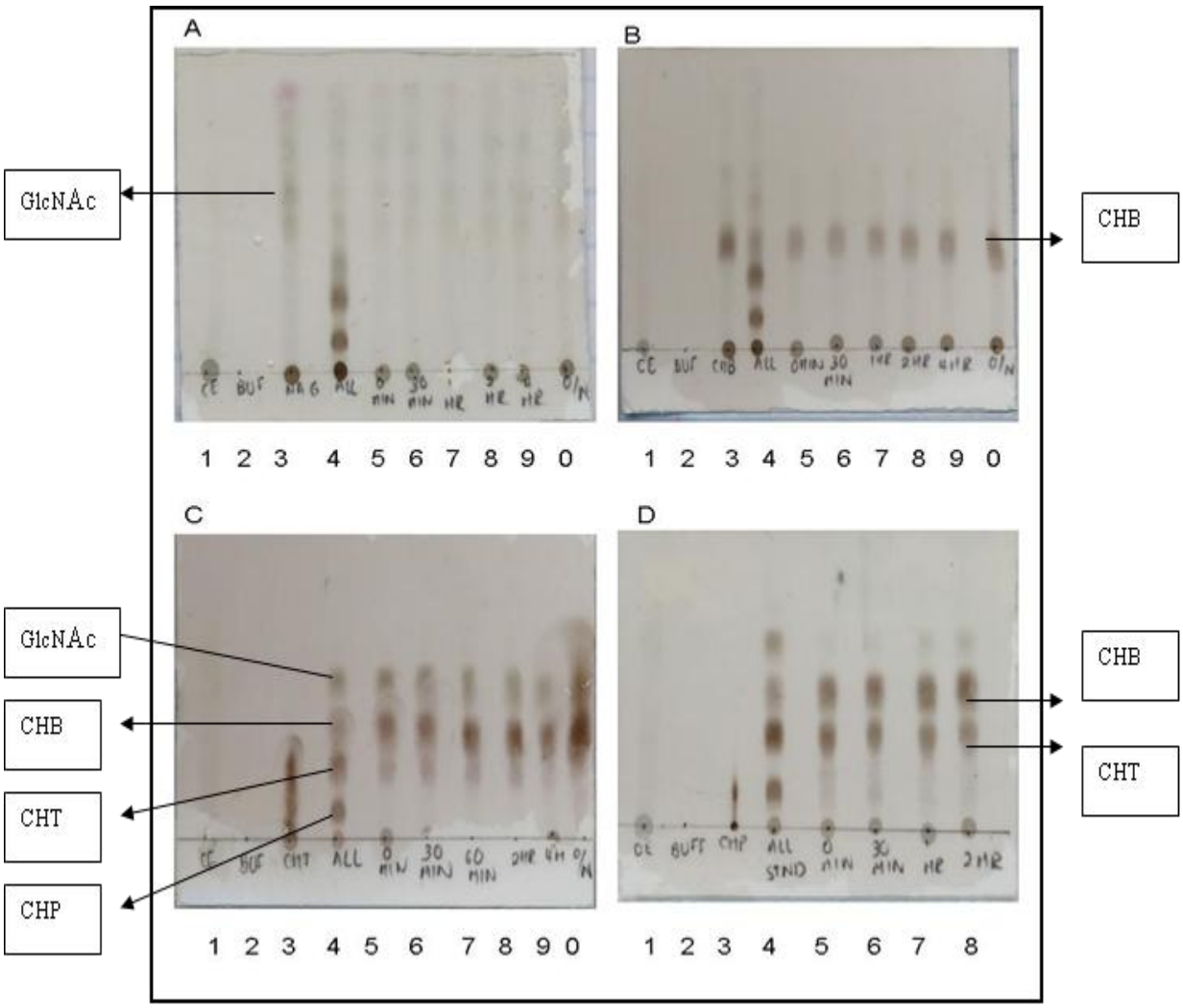


Figure 34. TLC analysis of strain PBY chitinase reaction products

Reactions mixtures contained 1.36 mM of the respective substrates together with 30 µg of the extracellular chitinase in a phosphate buffer environment (pH 7.2). Reactions were incubated at 30^{o}C for 0 min–16 hours. Enzyme-substrate reaction mixes were incubated at 30^{o}C for 0min–16 hours. The resultant analysed samples were run on Silica 60 TLC plates using an n-butanol: acetic acid: water solvent system (3:1:1 v/v/v). Once completed the TLC plates were air-dried prior to spraying using a solution of diphenylamine: aniline which was used for detecting the presence of reducing sugars. The sprayed plates were then subjected to heating at 180^{o}C for 2 to 5 minutes prior to the individual hydrolytic products becoming visible.

Plate A: Strain PBY incubation with GlcNAc with reaction product analysis done from 0 minutes up to and including 16 hours. A1–cell extract; A2–buffer control; A3–GlcNAc control; A4–all controls; A5–A0; reaction product at 0 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 16 hours.

Plate B: Strain PBY incubation with CHB with reaction product analysis done from 0 minutes up to and including 16 hours. B1–cell extract; B2–buffer control; B3–CHB control; B4–all controls; B5–B0 reaction product at 0 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 16 hours.

Plate C: Strain PBY incubation with CHT with reaction product analysis done from 0 minutes up to and including 16 hours. C1–cell extract; C2–buffer control; C3–CHT control; C4–all controls; C5-C0; reaction product at 0 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 16 hours.

Plate D: Strain PBY incubation with CHP with reaction product analysis done from 0 minutes up to and including 2 hours. D1–cell extract; D2–buffer control; D3–CHP control; D4–all controls; D5-D8; reaction product at 0 minutes, 30 minutes, 1 hour, 2 hours.

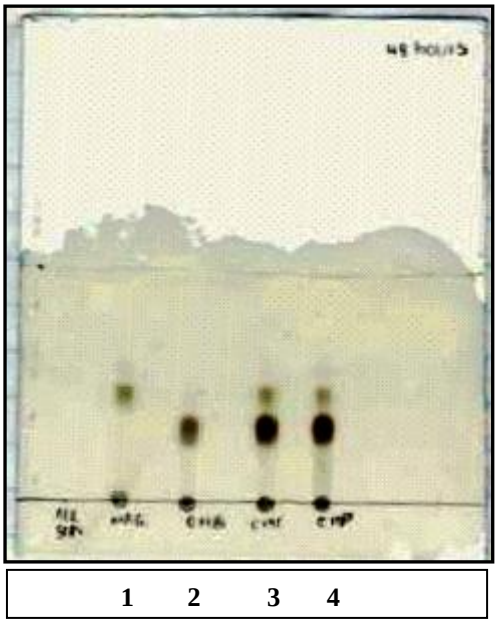


Figure 35. TLC analysis of strain PBY chitinase reaction after a 48 hours incubation period with CHT and CHP as substrate

All reactions mixtures contained 1.36 mM of substrate together with 30 µg extracellular chitinase protein with the reaction carried out in a phosphate buffer environment (pH 7.2).

Lane 1: GlcNAc control

Lane 2: CHB control

Lane 3: Strain PBY chitinase incubated with CHT for 48 hours at 30^{o}C. Reaction products formed include GlcNAc as well as CHB

Lane 4: Strain PBY chitinase incubated with CHP for 48 hours at 30^{o}C. Reaction products formed include GlcNAc as well as CHB

3.5 Conclusion

The kinetic constants obtained from the characterisation of the extracellular chitinase from strain PBY when compared to documented literature on *B. licheniformis* chitinases showed similarities in their functional properties. More specifically the V_{max} data of the purified chitinase when tested on 4-MU CHB was 1.344 µmol/min/mg, while reactions with CHT as substrate showed the V_{max} value to be 2.288 µmol/min/mg. Comparative V_{max} data from *B. licheniformis* using para-nitrophenol CHB as substrate showed that the V_{max} data ranged from 2.9-3.8 µmol/min/mg. The enzyme was found to hydrolyse longer chain chito-oligosaccharides at a more rapid rate than the shorter substrate counterparts possibly indicating that the enzyme-substrate complex undergoes dissociation more rapidly in the presence of longer chain substrates. The K_m value obtained for the varied length substrates is particularly interesting since it shows the affinity that the enzyme has for the substrate. A lower K_m value shows stronger enzyme binding to the substrate, therefore the data obtained from 4-MU CHT shows that the purified chitinase binds a trimeric substrate more strongly then a dimeric one.

The chitinase pH and temperature profile shows that the characterised chitinase is a thermostable enzyme with extended chitinase activity maintenance at temperatures between 10-50^{o}C (Figure 32). This is unique since the chitinase is produced by a mesophilic isolate. Data extrapolation from the enzyme stability study done shows a 50% loss of activity at 65^{o}C during an hour incubation period suggesting that temperature elevations beyond the critical 60^{o}C probably results in some form of structural changes in the enzymes. Thermal denaturation studies alone can indicate whether this denaturation is reversible or irreversible.

The substrate hydrolysis patterns obtained using varied length chito-oligosaccharides shows that the enzyme only hydrolysis substrates containing a minimum of three saccharide moieties. Substrates such as CHT as well as CHP were iteratively hydrolysed with CHP being hydrolysed first to CHT then to CHB and GlcNAc. The chitinase activity obtained using 4-MU CHB which contains two chito–saccharide residues would appear to be contradictory to the previous statement except for the

observation that the 4-MU moiety is possibly being recognised by the enzyme as another saccharide moiety thus allowing 4-MU CHB to be seen as a trimeric substrate.

The hydrolytic action of the extracellular endochitinase purified is in agreement with previous literature which shows that extracellular chitinases often hydrolyse large polymeric chitin substrates into smaller chito-saccharides such as GlcNAc as well as chitobioside which can easily traverse the cell membrane and be processed further by intracellular chitinolytic enzymes. The CHB formed as a product of chitinase activity once inside the cell most likely serves to induce transcriptional expression of the chitinase enzyme and thus increase production of chitinase for excretion outside of the cell. This, however, can only be verified by testing varied inducers on chitinase activity as well as testing the concentration of inducers on final chitinase activity.

It is therefore likely that the chitinase studied is an endo-acting chitinase which under the conditions produced is responsible for the hydrolysis of long chain chito-oligosaccharides into smaller size GlcNAc and CHB residues which are more easily transported easily into the cell through the cell membrane.

CHAPTER FOUR

DISCUSSION

4 CHAPTER FOUR - DISCUSSION

Chitinase enzymes are crucial for the biodegradation of chitin containing waste products from both aquatic as well as terrestrial environments. In terms of practical applications chitinases are often used in the preparation of protoplasts from fungal cells, as a protective agent against phytopathogenic fungi and in the production of biologically active compounds. Most chitinase producers reside in a select population of *Aeromonas* spp., *Bacillus* spp., *Chromobacterium* sp., *Serratia* spp., *Vibrio* spp., as well as *Streptomyces* spp. strains. Considering the vast potential of *Bacillus* spp. catalysts makes the selective isolation of chitinolytic microbes from this particular genus considerably favourable.

4.1 Cell enumeration and strain isolation

The *Bacillus* enrichment strategy tested for isolating chitinolytic strains from environmental samples resulted in the isolation of two chitinolytic strains. From the two isolated strains, strain PBY was selected as the strain of choice since it appeared to hydrolyse the chitin present on the minimal media plates at a much more rapid rate. Strain PBY was further cultured and taxonomically identified as being most alike to a *P. chitinolyticus* species from 16s rDNA hybridization studies done (> 99% sequence identity). Currently the only *Paenibacillus* strain with documented sequence data for cloned and purified chitinase gene/s is from *Paenibacillus ehimensis* EAG-53 which contains the following proteins chi80 (Q7X5L8), chi60 (Q7X5L9) and chi55 (Q7X5M0)**.**

4.2 Chitinase enzyme induction

From the view point of chitinase production and cell harvesting for enzyme purification, work was done using a minimal media comprising chitin, yeast extract as well as calcium chloride. This media was found to be adequate for induction of chitinase activity with sufficient activity for purification observed after 72 hours of incubation with agitation at 25^{o}C. Inadequate levels of chitinase activity were observed when cells were cultured on rich media containing chitin even after a week of incubation. This

suggests that the chitinase activity was most likely repressed by the presence of glucose in the media. This is in agreement with documented literature pertaining to the repressive nature of glucose towards chitinase activity induction in *Streptomyces lividans* as well as *Trichoderma harzianum*. The repression observed is most likely catabolite repression which in cells enables selective utilization of simple sugars hydrolysed by constitutively produced enzymes**.** In the absence of simple sugars, inducer substrate molecules such as CHB or other soluble chito-oligosaccharides increase expression of chitinolytic enzymes. In *S. lividans* chitinase activity induction involves transcriptional induction from the *chiA* promoter. The effect of glucose on enzyme inactivation was tested on a *T. harzianum* chitinase with 90-100% of chitinase activity maintained at concentrations used in repression studies. This again validates the hypothesis that repression occurs at a transcriptional level.

4.3 Extracellular chitinase purification

Enzyme purification was successfully achieved using a two-fold strategy of chitin affinity purification followed by anion EX chromatography which enabled the isolation and resolution of the chitinolytic enzymes present in the CFE processed. The dominant extracellular chitinolytic enzyme having a pI of ~7.0 was purified and characterised further. Chitinase activity purification resulted in a 107 fold increase in activity with the overall chitinase yield being 7%. This yield, although low when compared to the final 15% chitinase yield obtained from *Bacillus* sp. BG 11, can be improved by adjusting the purification steps. An extension of the elution incubation period and an increase in incubation temperature during elution will most likely result in improved chitinase recovery. It is however important to note that the significance of a simple enzyme purification protocol is in its ease of use for facilitation of enzyme purification. Any recombinant expression and characterisation work in future will require a quick and effective protocol for purification of the enzyme of interest from the remaining native cell proteins.

4.4 Kinetic data from extracellular chitinase

Assaying for chitinolytic activity was done using varied length fluorogenic chitin substrates. Reaction rate data obtained showed the enzyme had a distinct preference for

longer chain substrates with no activity observed against 4-MU Glc (K_m value was 7.45 µM for 4-MU CHB and 5.72 µM for 4-MU CHT). This inability to hydrolyse substrates containing less then two sugar residues in size indicates the absolute requirement for the presence of three residues for binding and catalytic hydrolysis. Although activity was observed against 4-MU CHB (which comprises of two GlcNAc residues together with a 4-methylumbelliferyl moiety) the activity is likely to be due to the enzyme recognising the *O*-glycosyl linkage between the 4-methylumbelliferyl moiety and the remaining sugar residue and therefore recognising 4-MU CHB as a trimeric saccharide. The enzyme also lacks the ability to hydrolyse substrates from the non-reducing end of the sugar as noted by a lack of activity against 4-MU glucosaminide. It is therefore possible to say that the enzyme purified is not a hexosaminidase enzyme.

4.5 Two dimensional characterisation of the extracellular fraction

Two-dimensional characterisation of the CFE prior to purification showed the presence of multiple chitinolytic enzymes in the sample. The observed chitinolytic activity from zymography studies done using glycol chitin as substrate showed the presence of chitinolytic activity at pI values ~5.0 as well as pI ~7.0-9.0. This was once again verified when initial ion exchange studies done using a universal buffer showed the presence of two chitinase activity peaks at pH values of 5.0 and 7.0. Most documented microbial chitinases have pI values that range from 3.5-8.8, with some organisms having multiple chitinase isozymes. From a comparative viewpoint the chitinase pI values observed after focusing of the crude extracellular protein from strain PBY show that the pI values fall within the same documented range.

The presence of multiple chitinolytic enzymes/ isozymes in an organism is not uncommon. Work done on *Bacillus circulans* WL-12 showed the presence of six distinct chitinolytic enzymes with chitinase A1 and A2 and chitinase B1 and B2 postulated to have been derived from each other (Watanabe *et al.* 1990). More specifically the chitinase isozymes produced by *Bacillus circulans* WL-12 demonstrated pI values of 4.7, 4.5, 6.6, 5.9, 8.5 as well as 5.2. This is quite similar to the 2-D results obtained from the novel environmental isolate of strain PBY. It is therefore possible

that what is observed as multiple chitinolytic enzymes might be isozymes resulting from gene divergence in the strain being studied as well. The only way to confirm or negate this would be to complete N-terminal sequencing of the focused and purified protein spots. Maldi-Tof sequencing will also provide invaluable information on the novelty of the enzyme and allow for the amino acid sequence of the chitinase protein sample to be compared to other documented chitinase enzymes.

4.6 Temperature and pH effects on chitinase activity

In terms of temperature and pH profiling the characterised chitinase enzyme was found to be a mesophilic enzyme which functioned optimally at neutral pH conditions. The enzyme demonstrates a half life of 1 hour at 65^{o}C. A rapid decrease in chitinase activity at pH 5.5 and more acidic environments was observed with a 50% loss of activity noted between pH 7.0 and pH 5.5 (Figure 31). This most likely indicates a change in the ionization of either the acidic or basic residues involved in the catalytic activity. Considering that most chitinases function through a general acid catalysis mechanism and since the catalytic residues implicated in activity most commonly involve conserved glutamate and aspartate residues, a change in ionization would decrease activity or render the enzyme inactive. It is also possible that a change in pH may alter the shape and charge of both the enzyme and the substrate therefore resulting in altered substrate recognition or enzyme inactivation.

Typical standard free energies of activation of 15-70 kJ/M-1 generally result in an increase in activity rate of a factor of 1.2 to 2.5 for every 10^{o}C increase in temperature. This increase in activity brought about by an increase in enzyme-substrate collisions as well as an increase in energy input into endothermic reactions is shown by the increase in chitinase activity at temperatures between 10-20^{o}C (see Figure 33). The consistent maintenance of activity between 10-50^{o}C during temperature stability studies (Figure 32) even after incubation of the enzyme suspensions at the respective temperatures for an hour could indicate thermal stability of the enzyme. A resistance to structural unfolding in chitinases has been observed in chitinase 40 produced by *Streptomyces thermoviolaceus*, where proline residues present in the protein in the *cis*-conformation caused a slow relaxation of the protein and consequently showed a slow refolding of the

enzyme after thermal denaturation. This was largely due to the high activation energy needed for isomerisation (Pyrpassopoulos *et al.* 2006). The reversibility of the thermal denaturation however was not studied for the strain PBY chitinase and therefore no suggestion can be made on the permanence of the denaturation.

The observed rapid decrease in activity at temperatures above 50^{o}C (Figure 32) could imply that the energy for conformational change supplied as an increase in temperature enabled enzyme unfolding, or that covalent changes to the enzyme (this includes the de-amination of asparagine residues or non-covalent changes such as the rearrangement of protein chains) could be inactivating the enzyme.

Unlike thermophilic organisms, most mesophilic microorganisms such as those belonging to the genus *Vibrio* as well as the enzyme studied from strain PBY have chitinase enzymes that function optimally at temperatures of 20°C to 40°C. In terms of comparative studies, the observed temperature optima for the characterised chitinase from strain PBY does not differ significantly from those documented for *Bacillus circulans*, *Vibrio* sp. as well as *Vibrio alginolyticus.* The pH optimum however varies greatly from one organism to another with a noted observation that a single chitinase enzyme when tested against varied substrates showing markedly different pH optima. This is largely due to the ability of the enzyme to bind the chito-saccharide or the existence of another chitin binding domain on the enzyme. Therefore the pH optimum of the strain PBY chitinase is likely to alter if tested on another substrate.

4.7 Chitinase substrate hydrolysis pattern

Finally in terms of substrate hydrolysis patterns, TLC analysis of the enzyme reactions after co-incubation of the enzyme with GlcNAc, CHB, CHT as well as CHP showed that the predominant products appeared to be CHB and GlcNAc. Furthermore substrates such as GlcNAc as well as CHB were not hydrolysed even after a 24 hour incubation period. This suggests that the enzyme is unable to bind substrates which have fewer then three residues into its catalytic site. Considering the enzyme preference for longer substrates it is most likely to be an endochitinase with a cleft or groove active site. This active site topography would allow for random binding and hydrolysis of

polymeric substrates, whereas an enzyme having a pocket shape topography would only recognise a non-reducing extremity saccharide for hydrolysis. The active site is unlikely to have a tunnel topography since this has only been found in cellobiohydrolases. The hydrolytic action of the extracellular endochitinase purified is in agreement with previous literature which shows that extracellular chitinases often hydrolyse large polymeric chitin substrates into smaller chito-saccharide such as GlcNAc as well as chitobioside which can easily traverse the cell membrane and be processed further by intracellular chitinolytic enzymes. The CHB formed as a product of chitinase activity once inside the cell most likely serves to induce transcriptional expression of the chitinase enzyme and thus increase production of chitinase for excretion outside of the cell. This, however, can only be verified by testing varied inducers on chitinase activity as well as testing the concentration of inducers on final chitinase activity.

4.8 Future work

Further investigations on the chitinolytic enzymes produced by strain PBY could include:

- Analysis of the intracellular chitinolytic enzymes produced by strain PBY to ascertain whether an exochitinase within the cell is being produced to iteratively hydrolyse CHB.
- N-terminal sequencing of the multiple chitinolytic enzymes could be done to determine whether the observed enzymes are isozymes or structurally different enzymes.
- An improvement on the purification protocol to increase final chitinase yields looking at altering the binding and elution parameters initially tested in this study.
- Expression of the chitinase enzyme in an alternate host, such as *Escherichia coli*, to increase chitinase protein yields and to do additional enzymatic studies using site–directed mutagenesis to determine the effect of specific amino acid residues on chitinase enzyme activity.

- A study into the effective repressors as well as inducers of chitinase activity in strain PBY as well as concentration effects on activity.

4.9 Possible application for enzyme

The observed chito-saccharide products formed by the endochitinase (GlcNAc as well as CHB) could be used for both agricultural and industrial application. The incorporation of the catalytic products into fertiliser formulations could be of interest since this would serve to elicit pathogen response reactions resulting in chitinase production in the plant prior to fungal or nematodal infection. Products such as GlcNAc as well as CHB have nutraceutical benefits as well (precursors used in the preparation of heteropolysaccharides such as chondriotin and hyaluron production).

Chitinase enzymes also have the potential to perform transglycosylation reactions. A study into the possibility of glycosidic bond formation under non-aqueous reaction conditions should be undertaken to ascertain the profile of glycopeptides produced if transglycosylation is possible.

APPENDICES

5 APPENDICES

Appendix 1: Reaction rate calculation used

The reaction rate data documented in this dissertation is calculated using the raw data plotted together with a best fit curve that most accurately represents the data drawn. The curve data is then used to calculate the reaction rate values. In the example below the data is best represented by a linear trendline (see Figure 36). The equation is then used to calculate the reaction rate of the data in the following manner

1 The final absorbance (A_f) is subtracted from the initial absorbance (A_i) to give a Δ Absorbance change (in this case 835.46 FI)

2 The Δ Absorbance is halved to obtain a midpoint absorbance reading (426.73 FI)

3 The value obtained in step 2 is then subtracted from the initial absorbance reading (A_i) with the absorbance value substituted in the equation to calculate the corresponding time taken for ½ Δ Absorbance (2.563)

4 The reaction rate is then calculated by dividing ½ Δ Absorbance (426.73/2.563). The reaction rate is therefore 166.49 FI/min

Time (minutes)	Flourescence Intensity (440 nm)
0	0
1	161.405
2	330.195
3	499.36
4	665.105
5	853.465

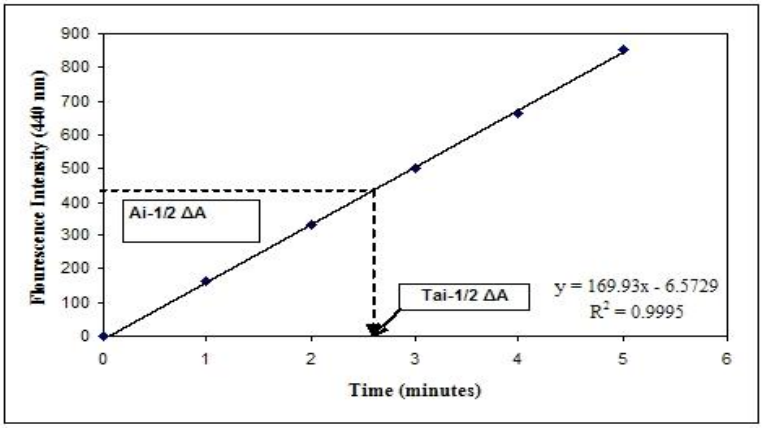


Figure 36. Use of best fit curve equation to calculate reaction rate

Data extrapolation using the reaction equation provides the reaction rate value data which in turn is converted to µmoles/ 4-MU/min using the 4-MU calibration curve shown in Figure 16 (see chapter 2)

Appendix 2: Strain PBY growth curve data and cell doubling time data on TSB

Cell doubling times were calculated using data from the region of the curve from which the growth appears to be logarithmic. A logarithmic plot (log_{10}) of the absorbance data ($A_{600/660}$ nm) was initially drawn to ascertain the data points of interest. The doubling time was then determined by re-plotting the data over the area of interest by converting the A_{600}/ A_{660} nm absorbance values into log_2 values. Using the equation of the curve, the inverse slope therefore indicated the time taken for the culture intensity to increase by density by 1 log_2 A_{600}/ A_{660} nm absorbance units. Since the data is log_2 a 1 absorbance unit change indicates that the absorbance has doubled (see chapter 2; section 2.5.1 for data plots). See Table 11 for the raw data used for determining the cell doubling time of strain PBY in TSB.

Table 11. Tabulated raw data used in the determination of the cell doubling time of strain PBY in TSB

Hours	Reading 1	Reading 2	Average	Log $_{10}$	Log $_2$	SD
1	0.015	0.016	0.015	-1.820		0.000
2	0.024	0.026	0.025	-1.610		0.001
3	0.032	0.036	0.034	-1.473		0.003
4	0.054	0.059	0.057	-1.246	-4.139	0.003
5	0.116	0.116	0.116	-0.936	-3.108	0.000
6	0.260	0.271	0.265	-0.576	-1.914	0.008
7	0.545	0.549	0.547	-0.262	-0.871	0.003

A loopful of *P. chitinolyticus* cells from an actively growing TSB agar plate was inoculated into 2 x 500 mL erlenmeyer flasks containing 100 mL TSB each. The flasks were incubated on an orbital shaker set at 220 rpm at 25^{o}C. Samples were taken hourly for 7 hours with each sample being assessed spectrophotometrically at 600 nm.

Appendix 3: Strain PBY cell size determination

Cells of strain PBY were cultured on TSB for 24 hours prior to cell measurement being done. Cell measurement was done using an Olympus BX-40 light microscope, with image capturing using an Olympus U-CMAD3 camera and size determination done using analySIS LS research software. Sample size determination was done using a 100X magnification objective (see Figure 37 for the microscopic cell sizing image). The tabulated statistical analysis of the data is shown in Table 12.

Table 12. Cell size determination with the arithmetic mean and statistical significance of data provided

Descriptive	Line length (µm)
Reading 1	5.34
Reading 2	4.56
Reading 3	3.09
Reading 4	5.13
Reading 5	4.60
Reading 6	4.25
Reading 7	3.74
Reading 8	4.01
Reading 9	4.01
Reading 10	3.89
Σ	4.26
Maximum	5.34
Minimum	3.09
SD	0.67
Data variance	0.45

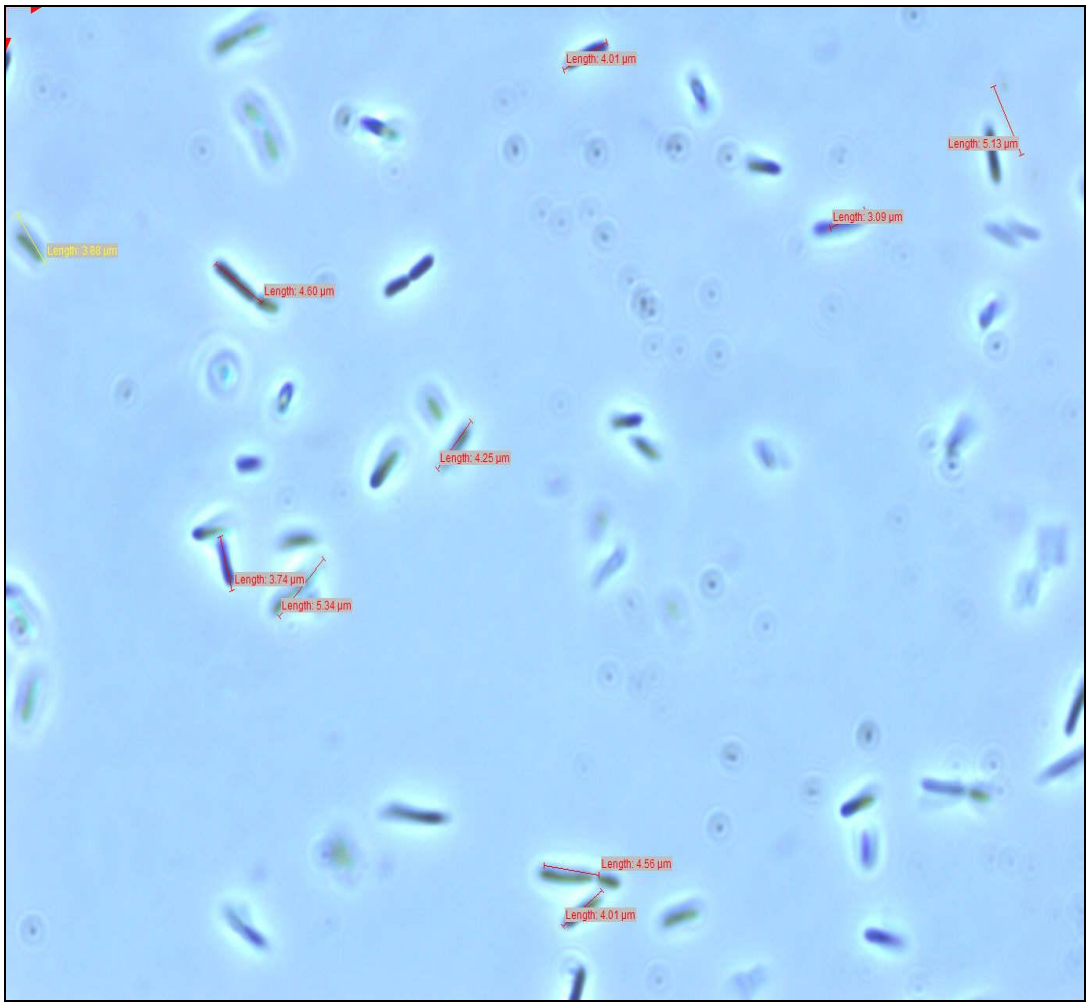


Figure 37. Microscopic image used to determine the average cell size of strain PBY

Appendix 4: Chitinase expression over 72 hours

Strain PBY cells were grown on minimal media for a period of three days with continuous agitation at 200 rpm on an orbital shaker. Activity levels were assessed using 4-MU CHB with fluorescence emission readings taken at 440 nm and excitation set at 360 nM. Slit widths of 10 mm were used and kinetic readings were assessed over 5 to 10 minutes (for data see Table 13 while the statistical analysis of the data can be seen in Table 14). Chitinase activity was expressed as the release of 1 µmole 4-MU per minute at 25^{o}C temperature. Protein quantification was done using the Bradford method (Bradford 1976). A bar graph representation of the data can be found in chapter 2; section 2.5.1; Figure 15).

Table 13. Chitinase activity on minimal media prior to harvesting

Sample Descriptive	Reaction rate (min1)	µmoles 4-MU	Dilution factor	[Protein]	Unit/ mL	Unit/mg protein
Sample 1.1	37.76	0.010	50.000	0.017	0.52	29.96
Sample 1.2	23.76	0.005	50.000	0.040	0.24	5.85
Sample 1.3	37.47	0.010	50.000	0.029	0.52	17.59
Sample 2.1	191.61	0.073	50.000	0.036	3.66	100.77
Sample 2.2	164.85	0.062	50.000	0.037	3.11	83.22
Sample 2.3	224.23	0.086	50.000	2.638	4.32	1.64
Sample 3.1	97.38	0.035	250.000	0.795	8.68	10.93
Sample 3.2	69.98	0.024	250.000	0.976	5.89	6.04
Sample 3.3	74.77	0.026	250.000	0.958	6.38	6.66

Samples 1.1 to 1.3 were readings obtained from triplicate flasks taken ~24hrs after incubation. Similarly samples 2.1 to 2.3 were taken ~48hrs after incubation, while samples 3.1 to 3.3 were taken ~72 hrs after incubation. All reactions were done using 10 µM CHB as substrate; chitinase activity was monitored as release of 4-MU in solution by fluorescence (excitation at 360 nm; emission at 440 nm).

Table 14. Statistical analysis of chitinase expression data

Sample descriptive	Reading1	Reading2	Reading 3	Average	SD	Data Variance
Sample 1	0.521	0.236	0.515	**0.424**	0.163	0.026
Sample 2	3.658	3.113	4.323	**3.698**	0.606	0.367
Sample 3	8.684	5.891	6.379	**6.985**	1.492	2.225

Appendix 5: 4-MU standard curve

A 50 mM stock solution of 4-MU was used to prepare the required samples needed for the standard curve preparation. The initial stock solution was prepared as follows: ~19.8 mg of 4-MU was weighed out and solubilised in 1-2 mL methanol prior to the sample being made up to 100 mL total volume in a 100 mL graduated volumetric flask using deionised water. Once completely mixed 400 µL was transferred into another 100 mL volumetric flask and made up to 100 mL using deionised water (200 nM stock solution). A series of dilutions then resulted into the preparation of the standards mentioned below (see Table 15 for raw data used in constructing the 4-MU standard curve). A substrate blank was also monitored to ascertain the stability over time of the substrate (see Figure 38 below). All samples once prepared were covered in foil to prevent photodecomposition. A diagrammatic representation of a typical 4-MU calibration curve can be seen in chapter 2; Figure 16.

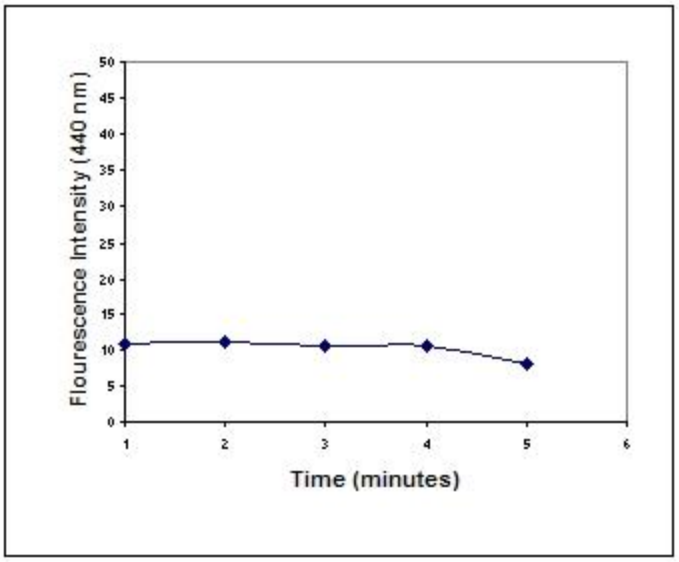


Figure 38. Diagrammatic representation of a typical 4-MU CHB substrate blank sample.

Fluorescence Analysis of the Substrate stability was done using 10 µM CHB made up to volume in 100 mM phosphate buffer, pH 7.0. Readings were taken every minute over a five minute incubation period, with the excitation wavelength set at 360 nm while the emission wavelength was 445 nm. The slit widths were set at 10 nm while the scan speed was 400 nm/second.

Table 15. Fluorescence data of individual 4-MU concentrations in solution for preparation of a 4-MU standard curve for chitinase activity quantification

Fluorescence intensity using 13.88 nM 4-MU		**Σ**	**SD**	**Data variance**
60.67	54.77			
58.38	53.57			
57.21	54.7			
57.62	53.39			
59.39	54.05	**56.375**	2.611	**6.82**

Fluorescence intensity using 27.75 nM 4-MU		**Σ**	**SD**	**Data variance**
97.11	95.04			
99.1	96.46			
99.25	96.45			
100.24	95.45			
101.64	95.04	**97.57**	2.33	**5.43**

Fluorescence intensity using 55 nM 4-MU		**Σ**	**SD**	**Data variance**
169.02	148.86			
168.88	147.34			
175.44	150.45			
174.03	149.13			
177.73	150.02	**161.09**	12.87	

Fluorescence intensity using 83.25 nM 4-MU		**Σ**	**SD**	**Data variance**
236.59	227.86			
233.27	229.97			
235.52	231.59			
237.79	232.48			
238.01	231.28	**233.43**	3.44	**11.81**

Fluorescence intensity using 111 nM 4-MU		**Σ**	**SD**	**Data variance**
294.44	290.57			
296.93	296.37			
297.8	296.14			
310.34	296.35			
301.76	300.66	**298.14**	5.28	**27.91**

Fluorescence intensity using 194.25 nM	**Σ**	**SD**	**Data variance**

4-MU				
502.6	511.06			
507.22	512.13			
506.74	516.6			
535.97	513.3			
510.97	514.22	**513.08**	9.01	**81.27**

Fluorescence intensity using 222 nM 4-MU		**Σ**	**SD**	**Data variance**
551.66	547.05			
558.79	555.11			
559.63	558.04			
560.79	560.7			
562.46	557.89	**577.21**	,4.72	**22.28**

Appendix 6: Ion Exchange Studies

Anion EX resins**:**

The HiTrap DEAE FF column specifications are as follows: bead size 45 µm – 165 µm; bead structure – 4% highly cross-linked agarose; weak anionic resin; dynamic binding capability – 43 mg BSA/ mL gel; the HiTrap QFF column specifications are as follows: bead size 45 µm – 165 µm; bead structure – 6% highly cross-linked agarose; strong anionic resin; dynamic binding capability – 120 mg HSA/ mL gel; the HiTrap Q Sepharose XL column specifications are as follows: bead size 45 µm – 165 µm; bead structure – 6% highly cross-linked agarose with bound dextran; strong anionic resin; dynamic binding capability – > 130 mg BSA/ mL gel; the HiTrap ANX FF column specifications are as follows: bead size 45 µm – 165 µm; bead structure – 4% highly cross-linked agarose; weak anionic resin; dynamic binding capability – 43 mg BSA/ mL gel (see Figure 39 to Figure 44 for strain PBY purification graph).

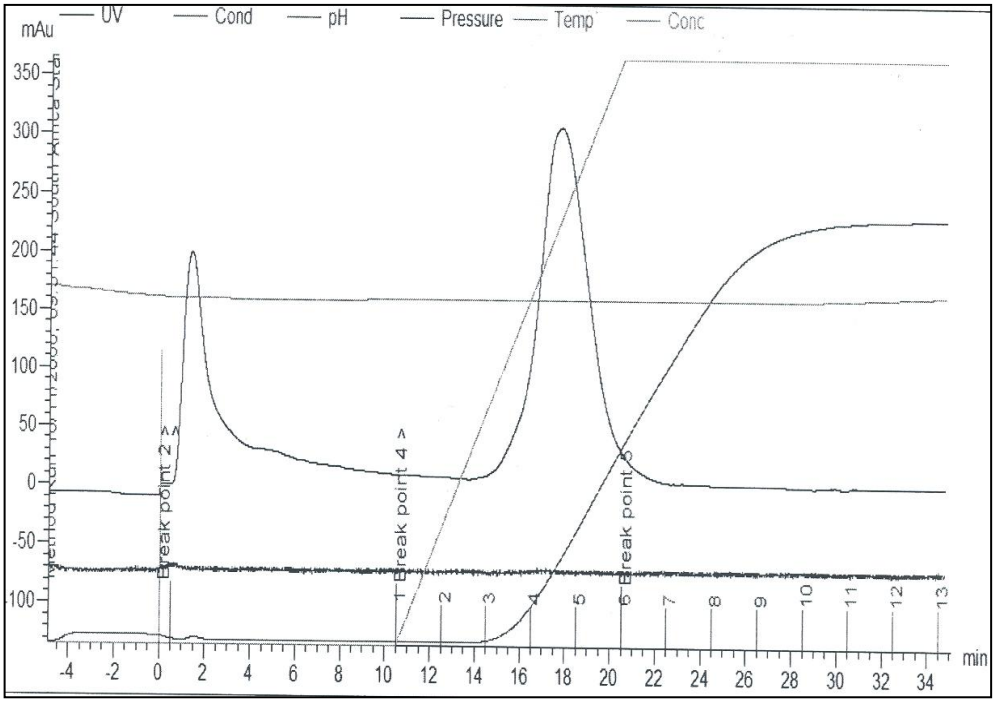


Figure 39. Anion EX chromatography of strain PBY CFE using DEAE FF column

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The DEAE FF column specifications are as follows: bead size 45 µm – 165 µm; bead structure – 4% highly cross-linked agarose; weak anionic resin; dynamic binding capability – 43 mg BSA/ mL gel. **Breakpoint 2** – sample injection from loop onto column, **breakpoint 3**-excess protein to waste detection (unbound protein), **breakpoint 4** – commencement of elution by injection of elution buffer (1M NaCl in 20 mM Tris (pH 9.0)), **breakpoint 5** – washing of column using start buffer (20 mM Tris Cl (pH 9.0)).

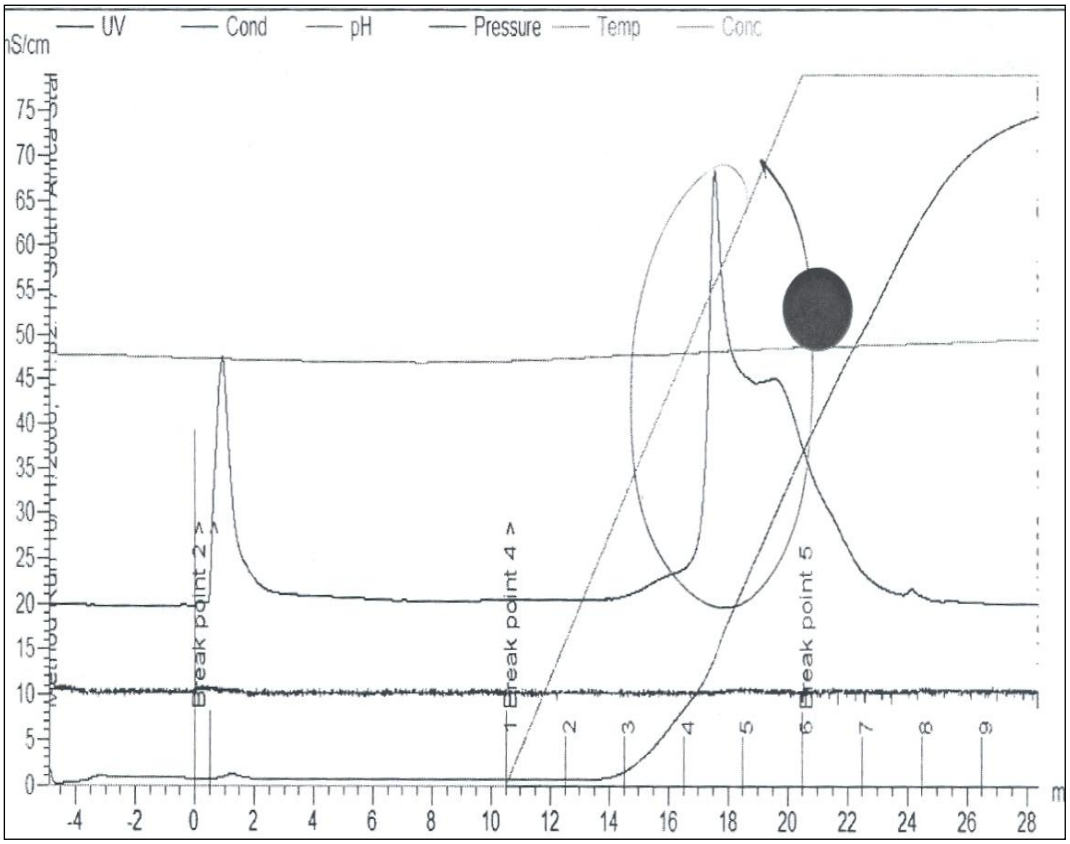


Figure 40. Anion EX chromatography of strain PBY CFE using HiTrap QXL column

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The Q Sepharose XL column specifications are as follows: bead size 45 µm–165 µm; bead structure–6% highly cross-linked agarose with bound dextran; strong anionic resin; dynamic binding capability– >130 mg BSA/ mL gel. **Breakpoint 2**–sample injection from loop onto column, **breakpoint 3**-excess protein to waste detection (unbound protein), **breakpoint 4**–commencement of elution by injection of elution buffer (1M NaCl in 20 mM Tris (pH 9.0)), **breakpoint 5**–washing of column using start buffer (20 mM Tris (pH 9.0)).

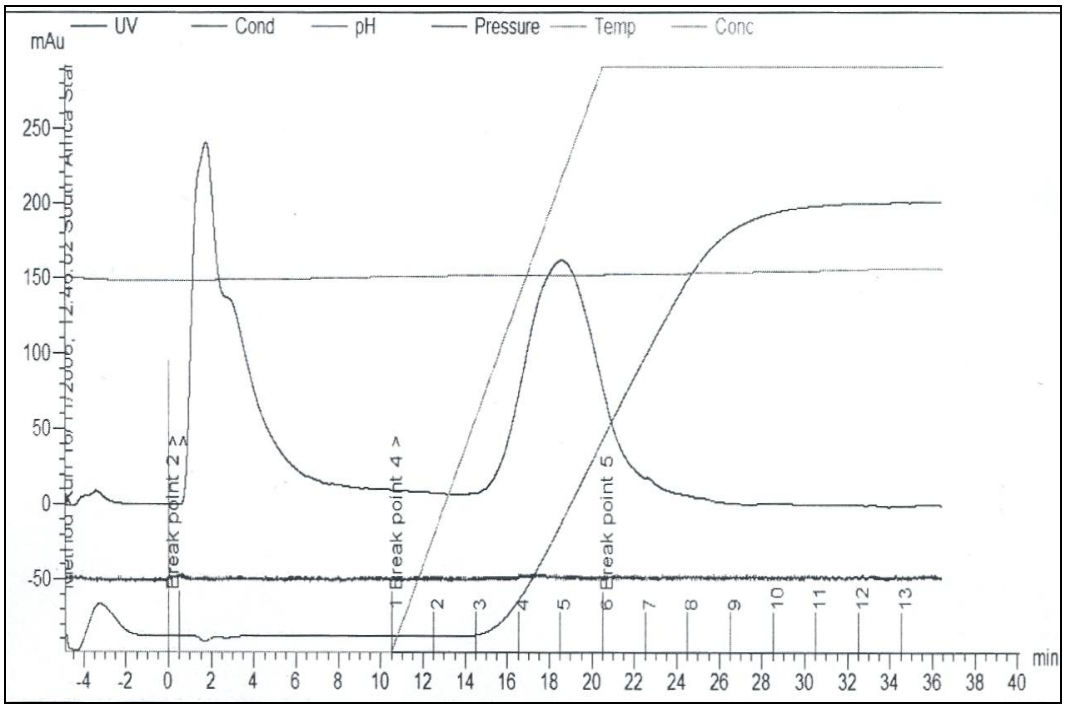


Figure 41. Anion EX chromatography of strain PBY CFE using ANX FF column

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The ANX FF column specifications are as follows: bead size 45 µm–165 µm; bead structure–4% highly cross-linked agarose; weak anionic resin; dynamic binding capability–43 mg BSA/ mL gel. **Breakpoint 2**–sample injection from loop onto column, **breakpoint 3**-excess protein to waste detection (unbound protein), **breakpoint 4**–commencement of elution by injection of elution buffer (1M NaCl in 20 mM Tris (pH 9.0)), **breakpoint 5**–washing of column using start buffer (20 mM Tris (pH 9.0)).

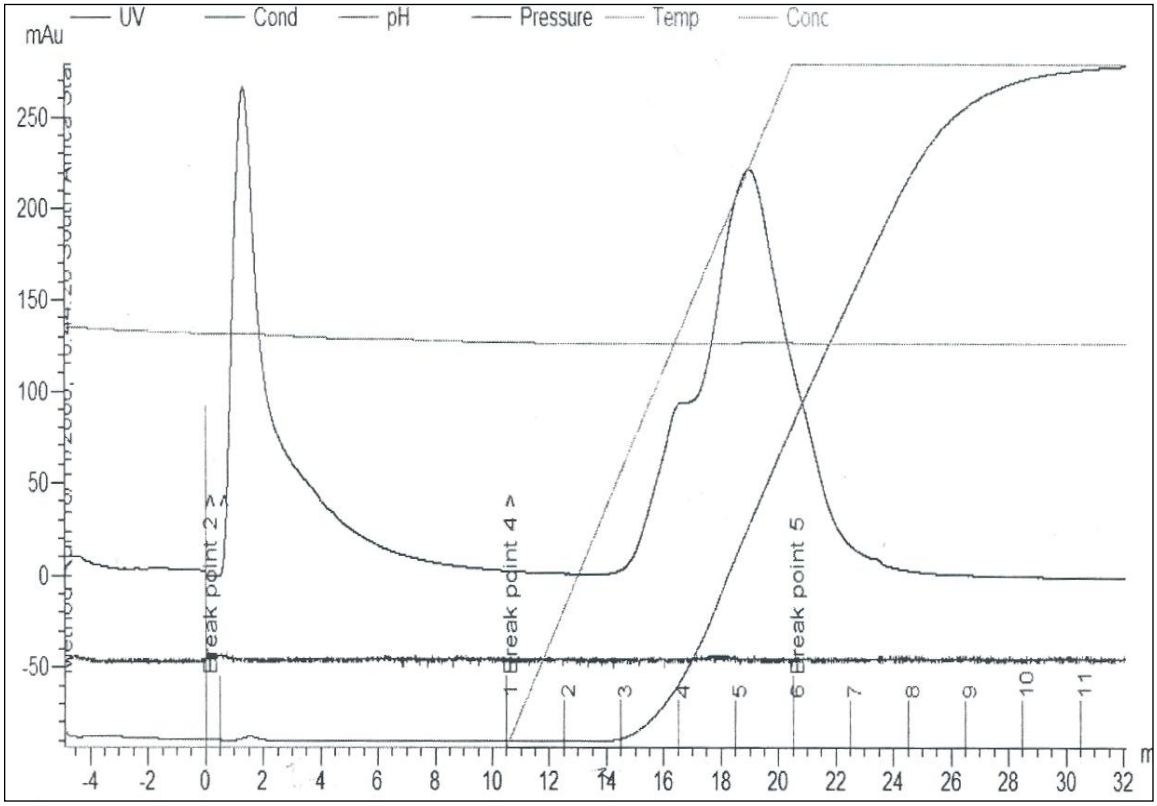


Figure 42**.** Anion EX chromatography of strain PBY CFE using QFF column

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The QFF column specifications are as follows: bead size 45 µm–165 µm; bead structure–6% highly cross-linked agarose; strong anionic resin; dynamic binding capability–120 mg HSA/ mL gel. Breakpoint 2-sample injection from loop onto column, breakpoint 3-excess protein to waste detection (unbound protein), breakpoint 4–commencement of elution by injection of elution buffer (1M NaCl in 20 mM Tris (pH 9.0)), breakpoint 5–washing of column using start buffer (20 mM Tris (pH 9.0).

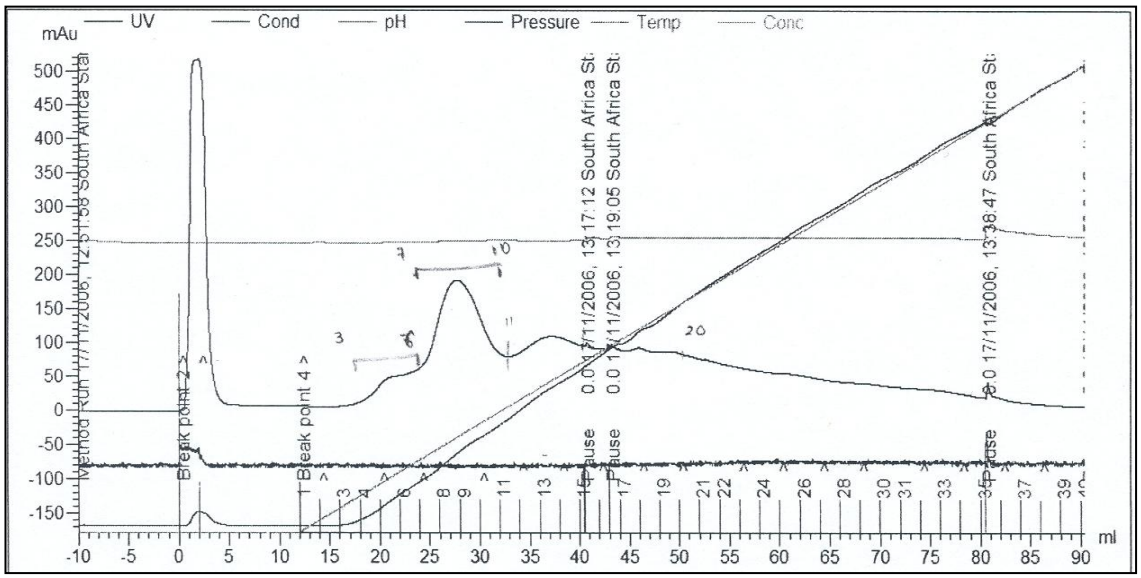


Figure 43. Shallow gradient protein elution on HiTrap QXL column using a 1M NaCl elution buffer.

Approximately 4mg protein was injected onto the column. Peak resolution was attempted using a modified run protocol. Fractions 3–6, 7–10 and 11–20 were combined and assayed for protein content using the Bradford method with activity being assayed using 4-MU CHB.

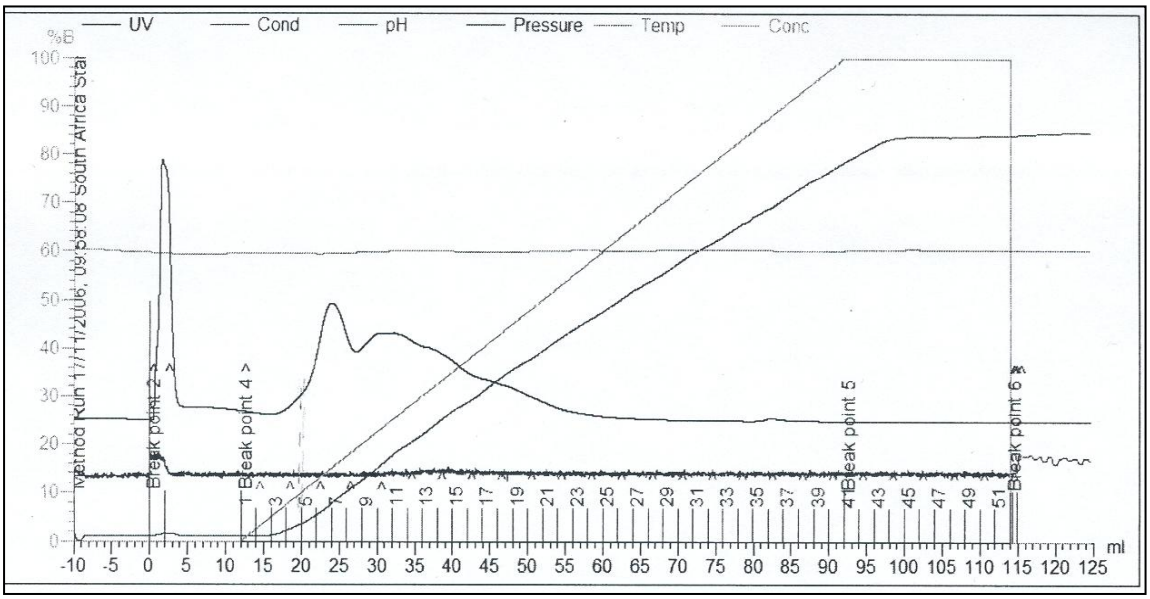


Figure 44. Shallow gradient protein elution on HiTrap QXL column using a 0.5M NaCl elution buffer**.**

Approximately 4mg protein was injected onto the column. Peak resolution was attempted using a modified run protocol. Fractions 4–7, 8–10 and 11–20 were combined and assayed for protein content using the Bradford method with activity being assayed using 4-MU CHB.

The HiTrap SPFF column specifications are as follows: bead size 45 µm–165 µm; bead structure– 6% highly cross-linked agarose; strong cationic resin; dynamic binding capability–70 mg ribonuclease A/ mL gel; the HiTrap CMFF column specifications are as follows: bead size 45 µm–165 µm; bead structure-6% highly cross-linked agarose; weak cationic resin; dynamic binding capability–50 mg ribonuclease A/ mL gel; the HiTrap SPXL column specifications are as follows: bead size 45 µm–165 µm; bead structure–6% highly cross-linked agarose with bound dextran; strong cationic resin; dynamic binding capability–>160 mg lysozyme/ mL gel (see Figure 45 to Figure 47 for strain PBY purification graphs).

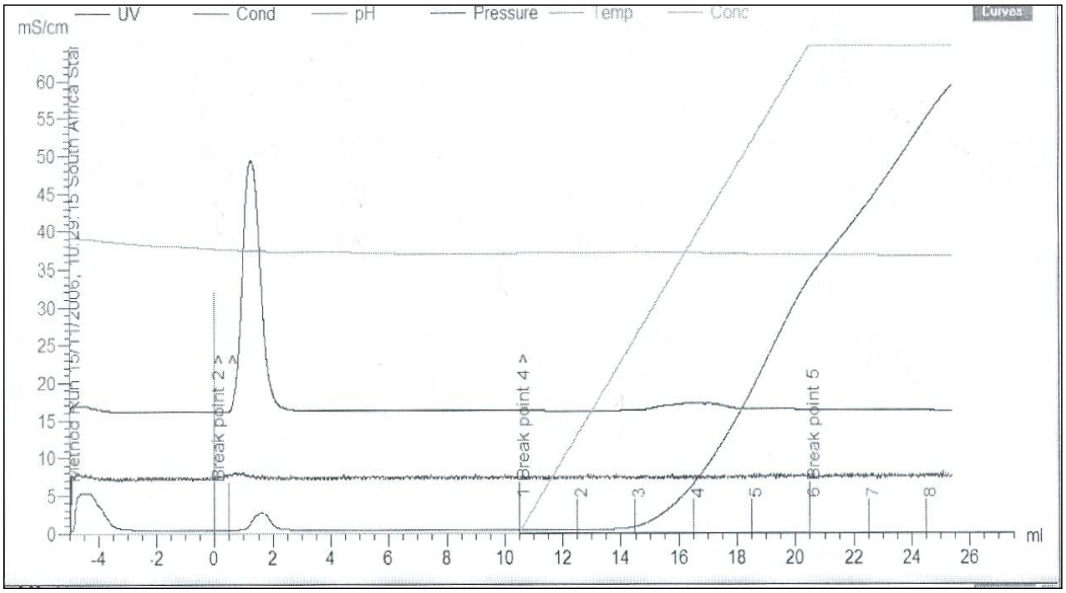


Figure 45. Cation EX chromatography of strain PBY CFE using HiTrap SPFF

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The SPFF column specifications are as follows: bead size 45 µm–165 µm; bead structure–6% highly cross-linked agarose; strong cationic resin; dynamic binding capability–70 mg ribonuclease A/ mL gel. **Breakpoint 2**–sample injection from loop onto column, **breakpoint 3**-excess protein to waste detection (unbound protein), **breakpoint 4**–commencement of elution by injection of elution buffer (1M NaCl in 20 mM MES (pH 6.0)), **breakpoint 5**–washing of column using start buffer (20 mM MES (pH 6.0)).

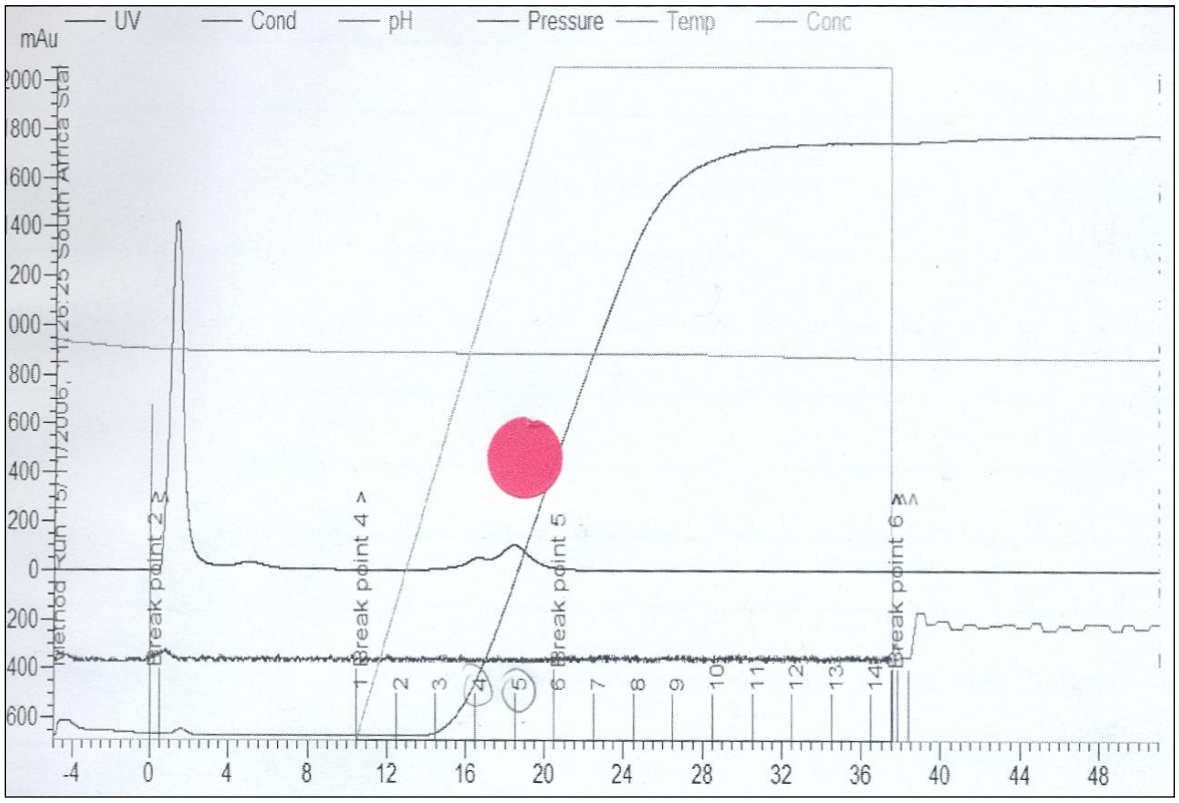


Figure 46. Cation EX chromatography of strain PBY CFE using HiTrap CMFF

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The CMFF column specifications are as follows: bead size 45 µm–165 µm; bead structure–6% highly cross-linked agarose; weak cationic resin; dynamic binding capability–50 mg ribonuclease A/ mL gel. **Breakpoint 2**–sample injection from loop onto column, **breakpoint 3**-excess protein to waste detection (unbound protein), **breakpoint 4**–commencement of elution by injection of elution buffer (1M NaCl in 20 mM MES (pH 6.0)), **breakpoint 5**-washing of column using start buffer (20 mM MES (pH 6.0).

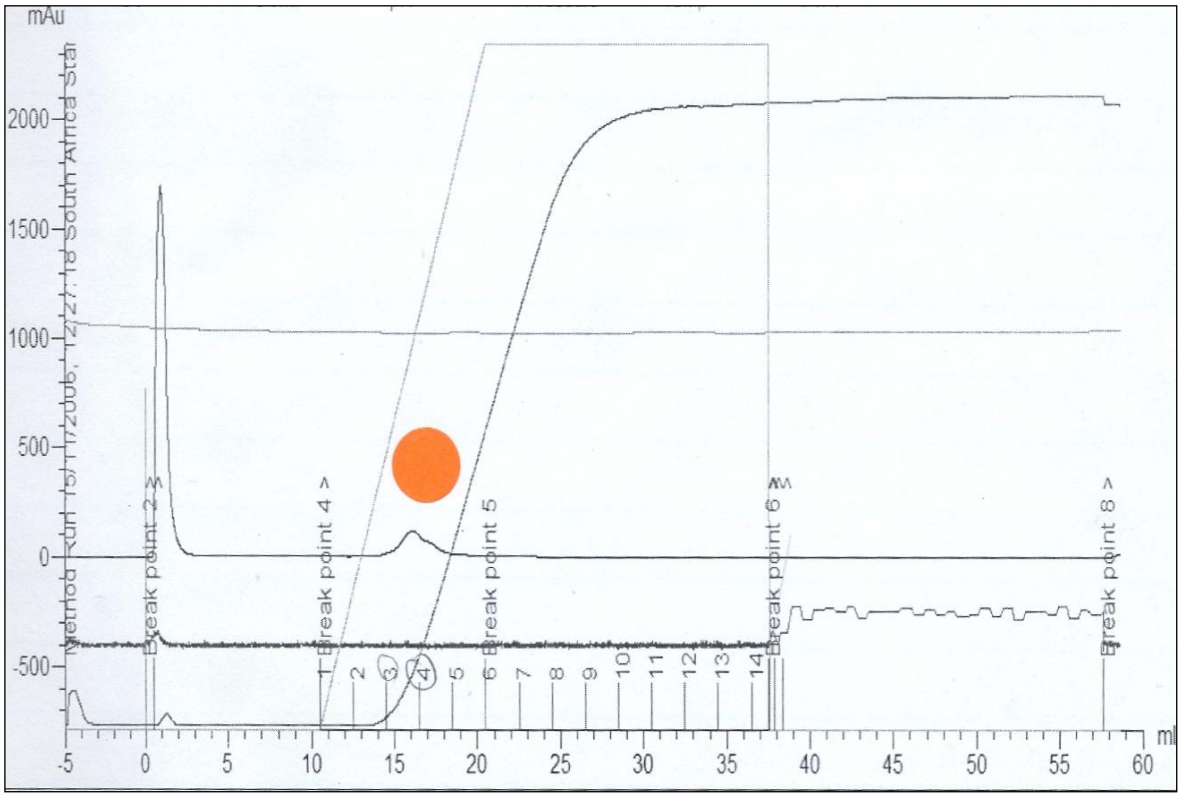


Figure 47. Cation EX chromatography of strain PBY CFE using HiTrap SPXL

Approximately 1 mL of the crude CFE (containing 3.8 mg protein/ mL) was injected and columned using the AKTA prime plus protein purification system. The HiTrap SPXL column specifications are as follows: bead size 45 µm–165 µm; bead structure–6% highly cross-linked agarose with bound dextran; strong cationic resin; dynamic binding capability–>160 mg lysozyme/ mL gel. **Breakpoint 2**–sample injection from loop onto column, **breakpoint 3**-excess protein to waste detection (unbound protein), **breakpoint 4**–commencement of elution by injection of elution buffer (1M NaCl in 20 mM MES (pH 6.0)), **breakpoint 5**–washing of column using start buffer (20 mM MES (pH 6.0)).

Appendix 7: SDS-PAGE confirmation of purity

Visual confirmation of chitinase purity and molecular mass determination was done using SDS-PAGE on a 10% acrylamide vertical slab gel (10 x 8cm). The CFE; concentrate after diafiltration, affinity purified chitinase sample as well as the purified chitinase after anion EX chromatography was run on an SDS-PAGE gel using the following protocol: SDS-PAGE sample buffer (0.0625 M Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue) was added to the sample (1:3), after which sample was boiled at 95ºC for 5 minutes. The 10% acrylamide separating gel (0.375 M Tris, pH 8.8, 0.1% SDS, 10% acrylamide) and 4% stacking gel (0.125 M Tris, pH 6.8, 0.1% SDS, 4% acrylamide) was prepared. Polymerisation of both the separating and stacking gels commenced by the addition of 0.05% ammonium persulphate and 0.005% $_{N}$, $_{N}^{I}$, $_{N}^{II}$, $_{N}^{III}$-tetramethylethylenediamine (TEMED). Size determination was done using a Fermentas protein molecular weight marker (SM 0431). The zymogram gel contained 0.1% (w/v) glycol chitin together with 0.8% low melting temperature agarose made up to volume using a 200 mM Tris buffer, pH 7.0.

The SDS-PAGE running buffer consisted of 25 mM Tris, 192 mM glycine and 1% SDS. The gel was resolved for at least one hour at 200 V. The staining of the polyacrylamide gels was performed using Coomassie stain (40% methanol, 0.7% acetic acid, 0.075% Coomassie dye) and destained using Coomassie destain (40% methanol, 0.7% acetic acid). The zymogram gel was stained using a 1% Congo-red solution made up in deionised water, while destaining was done using a 1 M NaCl solution. To enable easier viewing of areas of hydrolysis, acidification of the gel was done using a 0.2 M HCl solution which converted the red background to blue.

Appendix 8: Kinetic data using 4-MU CHB and 4-MU CHT

Leonor Michaelis and Maud Menten (1913) determined that the initial rate or velocity of catalysis of an enzyme varied hyperbolically with substrate concentration (Voet and Voet 1995). The initial rate increased with an increase in substrate concentration to a point where it would reach maximum velocity (V_{max}). At low substrate concentrations the initial rate was determined to be proportional to the substrate concentration, referred to as first order kinetics. At high substrate concentrations the initial rate was found to be independent of the substrate concentration, referred to as saturation or zero order kinetics. Michaelis and Menten derived a mathematical equation to express the relationship between the initial rate and substrate concentration:

$$V_o = \frac{V_{max}[S]}{K_m + [S]}$$

Where V_o is the initial rate, V_{max} is the maximal velocity of the reaction and K_m is the Michaelis-Menten constant. The K_m value was found to be equal to the substrate concentration at half the maximal velocity of the reaction, and was shown to be independent of the enzyme concentration and a characteristic of the system (Wilson and Walker 1995).

By definition the K_m value is a measure of the enzymes substrate affinity or the binding strength of the enzyme and its substrate. V_{max} is the limiting value of the initial velocity. The turnover number is an expression of the maximum number of moles of substrate that 1 mole of enzyme can convert to product in 1 second; it is the rate of the catalytic process or a measure of catalytic activity. The specificity constant (k_{cat}/K_m) is a measure of the relative reaction rate at low substrate concentrations; it is a second order rate constant that is a direct measure of the efficiency of the enzyme (Wilson and Walker 1995).

The kinetic data used for plotting of the chitinase enzyme kinetics with substrates 4-MU CHB as well as 4-MU CHT can be seen in Table 16 to Table 19).

Table 16. Chitinase activity (unit activity) per milligram protein using 4-MU CHB as substrate

N	µM 4-MU CHB	Reaction rate (min-1)	µmoles 4-MU	Dilution factor (*50)	Unit/ mg protein
1	1	26.06	0.0057	50	7.00
2	2	46.11	0.0138	50	17.25
3	5	70.52	0.0238	50	29.75
4	8	93.89	0.0331	50	41.25
5	10	109.13	0.0395	50	49.5
6	12	129.65	0.0479	50	60.00
7	15	144.95	0.0541	50	67.75
8	20	166.50	0.0629	50	78.75
9	30	188.54	0.0719	50	90.00
10	40	172.73	0.0656	50	81.75

Spreadsheet used for calculating chitinase activity (unit activity) per milligram protein. Reaction rate data was used to determine the 4-MU nmoles in solution concentration. This was then converted to µM values and multiplied by the dilution factor used to obtain unit activity of enzyme (µmol 4-MU /min). This was finally multiplied by a factor of 25 to get unit activity per milligram of enzyme.

Table 17. Data employed in preparing kinetic plots used for obtaining V_{max} and K_m values using 4-MU CHB as substrate

Michaelis-Menten		**Lineweaver-Burk**		**Hanes-Woolf**		**Eadie-Hofstee**	
X-axis	**Y-axis**	**X-axis**	**Y-axis**	**X-axis**	**Y-axis**	**X-axis**	**Y-axis**
[4-MU CHB]	Reaction rate (min)	[1/4-MU CHB]	1/Reaction rate (min^{1})	[4-MU CHB]	[4-MU CHB]/ Reaction rate (min	Reaction rate (min)/[4-MU CHB]	Reaction rate (min)
1	26.06	1.000	0.038	1	0.038	679.12	26.06
2	46.11	0.500	0.022	2	0.022	1062.84	46.11
5	70.52	0.200	0.014	5	0.014	994.61	70.52
8	93.89	0.125	0.011	8	0.011	1101.92	93.89
10	109.13	0.100	0.009	10	0.009	1190.94	109.13
12	129.65	0.083	0.008	12	0.008	1400.76	129.65
15	144.95	0.067	0.007	15	0.007	1400.74	144.95
20	166.50	0.050	0.006	20	0.006	1386.05	166.50
30	188.54	0.033	0.005	30	0.005	1184.91	188.54
40	172.73	0.025	0.006	40	0.006	745.89	172.73

Table 18**.** Chitinase activity (unit activity) per milligram protein using 4-MU CHT as substrate

N	µM CHT	Reaction rate (min-1)	µmoles 4-MU	Dilution factor	Unit Activity
1	1	54.75	0.0056	50	7.06
2	2	90.01	0.0138	50	17.25
3	5	154.85	0.0238	50	29.75
4	8	192.21	0.0331	50	41.37
5	10	216.57	0.0395	50	49.37
6	12	226.74	0.0479	50	59.87
7	15	252.89	0.0541	50	67.62
8	20	291.8	0.0629	50	78.62
9	30	215.19	0.0719	50	89.89

Spreadsheet used for calculating chitinase activity (unit activity) per milligram protein. Reaction rate data was used to determine the 4-MU µmoles in solution concentration (using the 4-MU regression curve (Figure 1). This was then converted to µM values and multiplied by the dilution factor used to obtain unit activity of enzyme (µmol 4-MU / min). This was finally multiplied by a factor of 25 to get unit activity per milligram of enzyme.

Table 19. Data employed in preparing kinetic plots used for obtaining V_{max} and K_m values using 4-MU CHT as substrate

Michaelis-Menten		**Lineweaver-Burk**		**Hanes**		**Eadie-Hofstee**	
X-axis	**Y-axis**	**X-axis**	**Y-axis**	**X-axis**	**Y-axis**	**X-axis**	**Y-axis**
[4-MU CHT]	Reaction rate (min)	[1/4-MU CHT]	1/Reaction rate (min)	[4-MU CHT]	[4-MU CHT]/ Reaction rate (min)	Reaction rate (min)/[4-MU CHT]	Reaction rate (min)
1	54.75	1.000	0.018	1	0.018	54.750	54.75
2	90.01	0.500	0.011	2	0.022	45.005	90.01
5	154.85	0.200	0.006	5	0.032	30.970	154.85
8	192.21	0.125	0.005	8	0.042	24.026	192.21
10	216.57	0.100	0.005	10	0.046	21.657	216.57
12	226.74	0.083	0.004	12	0.053	18.895	226.74
15	252.89	0.067	0.004	15	0.059	16.859	252.89
20	291.80	0.050	0.003	20	0.069	14.590	219.80

Appendix 9: pH Profile

Enzyme activity is pH dependent since the activity is dependent on the ionisation state of the amino acids in the active site (Wilson and Walker 1995): (Voet and Voet 1995). Most proteins are only active within a narrow pH range, usually in the range of 5-9 (Wilson and Walker 1995); (Voet and Voet 1995).

A visible increase in 4-MU fluorescence was detected at increasing pH values (see Figure 48). However all data shown in Table 20 had the substrate blank values subtracted prior to the data being tabulated.

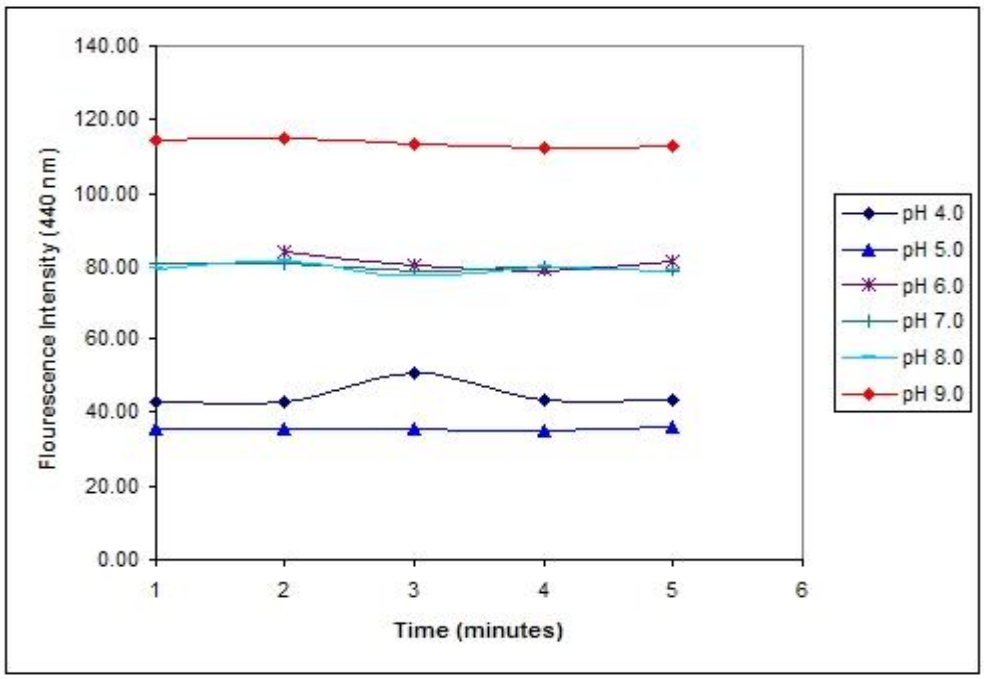


Figure 48. Fluorescence data for substrate blanks obtained using varied pH buffers solutions

Data was obtained using 10 µM CHB as substrate. The fluorescence data was obtained using an excitation wavelength of 360 nm while the emission wavelength was set as 440 nm. The slit widths were 10 nm while the scan speed was 400 nm/ second.

Table 20. pH Profile of strain PBY chitinase using 4-MU CHB as substrate

Universal buffer pH	Reaction rate- Σ	SD	Data variance	Relative % activity
pH 4.0	22.75	6.66	44.27	28.68
pH4.5	28.04	8.10	65.55	35.35
pH 5.0	32.96	0.47	0.22	41.54
pH5.5	40.50	0.01	0.002	51.05
pH6.0	74.24	1.03	1.06	93.58
pH6.5	67.69	0.95	0.90	85.32
pH7.0	76.98	2.28	5.18	97.03
pH7.5	79.34	1.51	2.27	100.00
pH8.0	64.59	0.43	0.19	81.42
pH8.5	46.31	0.21	0.04	58.37
pH9.0	27.79	1.08	1.17	27.79

Tabulated reaction rates obtained using a universal buffer solution pH adjusted to the required pH values ranging from pH 4.0 to pH 9.0. Substrate used was 10 µM CHB.

Appendix 10: Temperature profile

Enzymes are known to be sensitive to changes in temperature since the relationship between reaction rate of an enzyme and temperature is exponential. For every 10°C rise in temperature the rate of an enzyme reaction will double. At temperature ranges of between 40°C and 70°C most enzymes are denatured and lose their activity. Enzymes are known to display maximal activity at a temperature known as the temperature optimum of the enzyme (Wilson and Walker 1995). The tabulated data below shows the endpoint readings used for constructing the temperature profile of the characterised chitinase from strain PBY (Table 21).

Table 21. Chitinase enzyme profile data using 4-MU CHB as substrate

Temperature (^{O}C)	Endpoint reading- Σ	SD	Data variance	Relative % activity
10	196.06	76.71	588.3	75.19
20	219.85	5.92	35.03	84.32
30	259.73	6.80	46.27	99.61
40	260.74	25.50	650.52	100.00
50	204.46	4.19	17.58	78.41
60	168.41	10.38	107.75	64.59
70	157.04	33.27	1106.85	60.23
80	106.44	1.16	1.34	40.82

Chitinase reaction rates obtained from enzyme substrate reactions incubated at the required temperatures for 5 minutes prior to kinetic assaying

Appendix 11: Temperature stability

All enzyme thermal stability data shown in Table 22 have substrate blank values at the varied temperatures subtracted prior to data processing being done (see Figure 49 for a typical 4-MU CHB substrate blank).

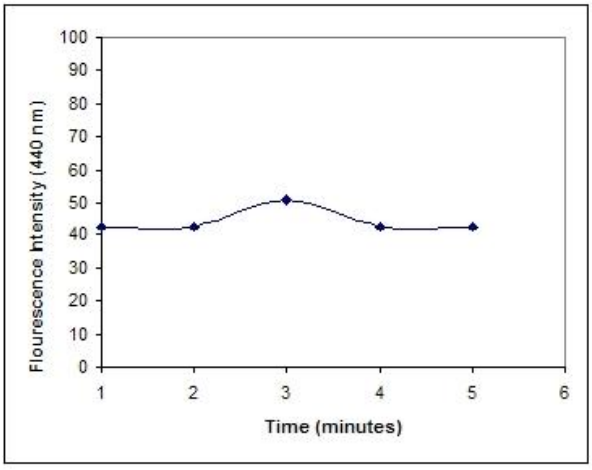


Figure 49. Representation of a typical 4-MU CHB substrate blank fluorescence intensity check over five minutes to determine the stability of the substrate in solution

The substrate blank contained 10 µM 4-MU CHB in 100 mM phosphate buffer. Readings were taken every minute over a five minute period. The fluorescence excitation wavelength was set at 360 nm while the emission wavelength was 440 nm. The slit widths were 10 nm while the scan speed was 400 nm/second.

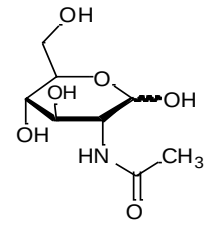
Table 22. Chitinase temperature stability kinetic data using 4-MU CHB as substrate

Temperature (^{O}C)	Reaction rate- Σ	SD	Data variance	Relative % activity
10	112.53	4.08	16.65	99.19
25	108.91	0.66	0.43	95.99
30	113.45	6.26	39.19	100.00
40	112.07	0.32	0.10	98.77
50	111.25	11.44	130.89	98.06
60	90.88	7.99	63.84	80.10
70	27.77	5.91	34.93	24.47
80	0.00	0.00	0.00	0.00
90	0.00	0.00	0.00	0.00

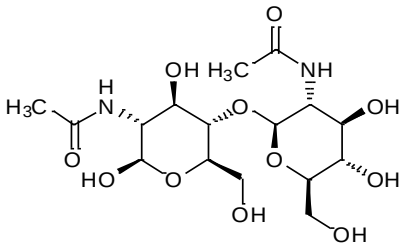
Tabulated reaction rates obtained from 0.2 mg/mL enzyme suspensions in buffer (100 mM phosphate buffer, pH 7.0) incubated at the required temperatures for 1 hour prior to assaying using 10 µM 4-MU CHB

Appendix 12: Structures of substrates used for ascertaining strain the extracellular chitinase hydrolytic patterns for strain PBY

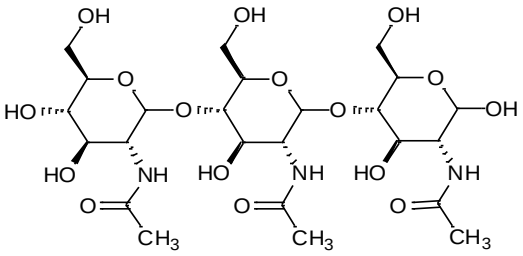
Structures of the varied length substrates used for determination of the enzyme substrate hydrolysis patterns are shown in Figure 50.



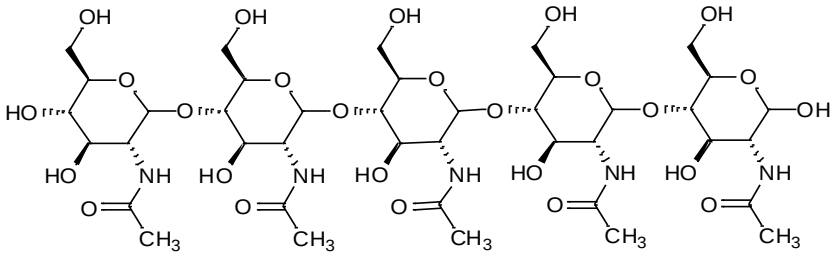
N-Acetyl-D-glucosamine



N,N'-Diacetylchitobiose



N,N',N''-Triacetylchitotriose



N,N',N",N'",N^{IV}-Pentacetylchitopentaose

Figure 50. Structures of compounds used for ascertaining chitinase hydrolytic pattern

Substrate concentrations used for assessing the nature of hydrolysis of the varied length chito-substrates was 1.36 mM of each respective substrate. The reaction contained 38.8 µg protein, with the reactions run in a 100 mM phosphate buffer environment (final reaction volume was 50 µL

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